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ROY WALDO MINER

MOLECULAR EVENTS IN DIFFERENTIATION RELATED TO SPECIFICITY OF CELL TYPE

BY

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Consulting Editor

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MOLECULAR EVENTS IN DIFFERENTIATION RELATED TO SPECIFICITY OF CELL TYPE*

Conference Chairman and Consulting Editor

H. CLARK DALTON

CONTENTS

* This series of papers is the result of a conference on *Molecular Events in Differentiation Related to Specificity of Cell Type* held by the Section of Biology of The New York Academy of Sciences, October 8 and 9, 1954.

INTRODUCTION

By H. Clark Dalton
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The term "molecular events" means different things to various people. As used in connection with this monograph, the words are intended to be taken in the broadest possible sense to include all kinds of processes from valence phenomena to the manifestations of long-range molecular forces; in short, to include anything that happens in differentiating cells at a submicroscopic level of activity. While, for this discussion, the type of molecular events is completely unspecified, the type of developmental events is limited to those aspects of differentiation which can be related to the origin and stabilization of cell types. This restriction has the purpose of limiting the choice of contributors to a practicable fraction of those investigators concerned with chemical aspects of embryology and, at the same time, of focusing attention on one of the deepest problems in biology.

The rationale of this approach rests on two basic assumptions. The first is that any attempt to understand the mechanisms of cellular differentiation must be based on the accurate description of molecular events associated with this crucial act of developing systems. In consequence, one key to the understanding of differentiation is the investigation of molecular events. The second assumption is that our progress in this attempt will be in proportion to the validity of our general theory, by means of which we approach our specific problems. It is, therefore, the aim of this publication, on the one hand, to assess our present ability to make the requisite description of the molecular events concerned and, on the other hand, to examine experimental methods and lines of thought that may profitably lead to a general theory of differentiation.

The papers presented can be subsumed under three headings. The first four are concerned with patterns of synthesis in differentiation. The next four deal with the immunobiological approach to problems of differentiation. The last three papers concern problems of structural organization.

Part I. Patterns of Synthesis in Differentiation

THE MOLECULAR BASIS OF THE FIRST HEART BEATS*

By James D. Ebert, R. A. Tolman, A. M. Mun† and J. F. Albright
Indiana University, Bloomington, Ind.

Introduction

The purpose of this account is to summarize and evaluate our knowledge of the sequence of events that culminate in the onset of contractility in the heart of the early chick embryo. Pertinent facts concerning cardiogenesis in other species will be presented for comparison. Three major topics will be considered:

(1) *The initial phases of heart formation.* Several lines of evidence pertaining to the distribution of heart-forming cells in the blastoderm at prestreak and early and definitive primitive streak stages, and their restriction to the heart-forming regions in the head process stage embryo will be analyzed, together with the role of tissue interactions in the development of the heart.

(2) *The development of the cardiac contractile proteins, myosin and actin.* Recent, previously unpublished findings concerning the immunochemistry and synthesis and distribution of cardiac actin will be emphasized. For clarity, considerable attention will be devoted to questions of the chemical and physical characteristics and antigenic specificity of actin.

(3) *The metabolism of the development of the heart.* This embraces a review of findings concerning enzymogenesis in relation to the synthesis of the contractile proteins and the onset of function. The role of the sarcosomes in cardiac metabolism will be stressed.

Initial Phases in Heart Formation

In the chick embryo, the heart, itself an unpaired structure, arises by the sequential fusion of paired primordia which, initially, lie widely separated on either side of the mid-line. The fusion of the cardiac primordia and the establishment of the early regional organization of the heart have been studied carefully in the chick by Patten (1922); in *Amblystoma* (Copenhaver, 1939); in *Fundulus* (Armstrong, 1931); and in a number of mammals (*e.g.* the rat; Goss, 1938). Patten (1949) has reviewed critically the problems arising from studies of the sequential formation of the primary regions of the heart. The early morphogenesis of the heart is foreshadowed by a precocious restriction of the heart-forming cells to relatively well-defined areas of the blastoderm at the head-process stage (Rawles, 1943). The evidence at hand, however, does not permit a clear understanding of the manner of origin of the heart-forming regions. The capacity for heart formation is widely distributed in the blastoderm at the prestreak stage, presumably in the epiblast, although there are no

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† Eigenmann Fellow in Zoology, 1954–1955.

data bearing directly on this point. Olivo (1928) reported that pulsating cardiac muscle develops in cultures of tissues taken from peripheral regions of previously unincubated chick embryos, but not in explants made from the central square millimeter of the blastoderm. From these findings he concluded, "Man kann also schlieszen, dasz am Ende des Furchungsprozesses die Herzanlage determiniert ist." Butler (1935) was able to demonstrate that heart muscle would develop from both anterior and posterior halves of the prestreak blastoderm when isolated and transplanted to the chorioallantoic membrane of host-chick embryos. Olivo's report of the failure of the central square millimeter of the embryo to produce heart muscle was not tested. All of the grafts made by Butler included peripheral material. Butler stated also that the posterior median quadrant of the prestreak embryo had the highest capacity for heart formation, a conclusion which cannot be regarded as more than tentative owing to the difficulty involved in orienting the prestreak blastoderm. Spratt (1942) has presented data in support of the idea that heart-forming potency is widespread initially, later becoming localized somewhere within the median portion of the posterior half of the blastoderm. Embryos were cut transversely at various levels into anterior and posterior pieces which were explanted to the surface of blood plasma-embryo extract clots *in vitro*. Pulsating heart masses developed in subcultures of the median portion of the anterior region of the prestreak blastoderm (incubated six to seven hours) in five cases and, in the posterior region, in nine cases. In explants taken at the beginning streak stage, cardiac tissue developed in the anterior half of the embryo in only one case, whereas explants of the posterior half produced heart in five cases. At the long-streak stage, heart developed only from the posterior half of the blastoderm. Neither Spratt's experiments nor the earlier investigations of Olivo (1928) and Butler (1935) offer any direct evidence as to whether heart-forming capacity is medially or bilaterally located at these early stages. The frequent occurrence of pulsating cardiac tissue on each side of the embryonic axis in these experiments may be attributed to mechanical separation of the heart primordia at the time at which fusion would have occurred. Rudnick (1938 a, b) concluded from a study of the differentiation in cultures of pieces of the blastoderm at short and definitive primitive streak and head-process stages that prospective heart-forming cells which, prior to invagination, might lie at or near the mid-line at the junction of head mesoderm and lateral plate, are early invaginated and migrate laterally to definitive positions on either side of the node. In a later review (1944) Rudnick suggests that these results indicate a migration of something very definite and may be taken as demonstrating a marked degree of autonomy. Although critical evidence is not available, Rudnick's data support the idea that, prior to invagination, the heart-forming materials lie in a single area and not in paired regions. Moreover, it is suggested that under the conditions of the experiments, the differentiation of cardiac muscle does not require interaction of the epiblast and endoderm. Explants were made both with and without endoderm. No significant differences between the two groups were observed.

By a series of precise experiments, Rawles (1943) has contributed information on the extent of the heart-forming regions in the head-process stage em-

bryo. Beginning approximately 0.2 mm. on each side of the mid-line, the areas extend laterally to approximately 0.8 mm. from the primitive pit. Anteriorally, the areas follow closely the tip of the head process, never extending beyond it. Their posterior extent is approximately 0.4 mm. from the pit. The areas have a center of strong differentiation tendency which is gradually diminished peripherally. The data do not indicate any striking asymmetrical differences in shape and extent of the areas. They do suggest, however, that the left side is superior to the right in so far as the capacity to produce grafts and hearts is concerned. It should be emphasized that the areas are considerably larger than the actual myocardial anlagen. Hence the heart-forming areas are to be regarded in the classical sense as areas of prospective potency and not of prospective fate. They are not "heart-specific" areas.

According to Bacon (1945), explants and gastrocoele implants of heart mesoderm from stage 11 of *Amblystoma* differentiate into normally curved and chambered hearts with rhythmic pulsations. Earlier than stage 11, no hearts are obtained. Contrary to the earlier concept of gradual determination of the heart (Stöhr, 1924, 1925; Ekman, 1929), Bacon concluded that complete primary organization of the heart occurs early in gastrulation. Removal of the archenteron floor from explants resulted in the development of two hearts, one at each of the lateral edges of the extirpation area. Moreover, explants of archenteron floor with presumptive mesoderm of stage 10 resulted in the appearance of rhythmically contracting tissue in the mesoderm. These findings led Bacon to conclude that "heart-organizing activity" is present in the endoderm of the archenteron floor. Comparable evidence does not exist for the chick. One direct experimental test, that of Rudnick (1938 a, b), who explanted pieces of the primitive streak stage embryo with and without endoderm and found no difference in the frequency of heart formation, points to a contrary conclusion. It must be pointed out, however, that the chick heart formed *in vitro* or in chorioallantoic transplants is not complete. It is generally a round or oval-shaped mass of histologically typical cardiac muscle, but lacks the characteristic chambered organization, a result that suggests the possible importance of extrinsic factors in bringing about a typical morphogenesis (Kumé, 1935; Rawles, 1943).

Thus a second problem is posed, namely: Given, that the heart-forming cells are distributed bilaterally in relatively well-defined regions of an apparently structureless sheet, how do the prospective cardiac cells differ from other cells in the same regions? In the chick embryo of seven somites (Hamburger and Hamilton, 1951; stage 9), the paired primordia have already begun to fuse. The events in cardiogenesis which occur between stages 5 and 10 merit close attention, for it is a period of intense activity. Local contractions have been recorded in the heart of the 9-somite embryo (Patten and Kramer, 1933). The onset of contractility has been correlated with the time at which significant histogenic changes have been recorded; *e.g.*, the first appearance of cross-striations at the 10-somite stage (Lewis, 1919) and the first detectable glycogen in the embryo of "about 28 hours" (Allen, 1919). Goss (1940) reported, from a carefully controlled series of observations on the developing rat heart, that myocardial elements which had exhibited their first contractions

showed neither definite myofibrils nor cross-striations. Szepsenwol (1946) reported similar findings from a study of the explanted chick heart. Although modern methods of ultrastructure analysis have not been brought to bear on the problem until quite recently, preliminary reports tend to support the earlier conclusion of Patten (1949), who wrote, "Thus it appears that the earliest contractions occur in cells that are but little specialized morphologically and which still lack the differentiated intracellular structures we are accustomed to think of as characteristic of muscle tissue. It is, nevertheless, significant that by the time their contractile activity has developed to any degree of efficiency, cross-banded myofibrils can be demonstrated . . . ." For example, Baud (1954), from a study of the fine structure of the developing chick heart, reported that although myocardial cells showed birefringent smooth myofibrils at or even before the onset of pulsations, the first sign of transverse striation, in the form of fibrils with a slightly heterogeneous birefringence, was not visible until "just before the appearance of the fast wave preceding the slow wave in the electrocardiogram." It is difficult to correlate the degree of heart development with the differentiation of cross-striations using the electrocardiographic information given. The youngest embryo from which Hoff, Kramer, DuBois, and Patten (1939) obtained satisfactory electrocardiographic records was a chick of 15 somites.

Synthesis and Distribution of the Contractile Proteins, Myosin and Actin, in the Development of the Heart

Myosin

We have seen that tests of potencies of small pieces of the early chick blastoderm through transplantation to the chorioallantoic membrane or to the surface of plasma clots *in vitro* show clearly that each isolated piece has a defined histogenic potency as shown by the frequency with which such pieces grow as grafts or isolates and by the amounts and kinds of tissues which grow in them. Particular structures develop only from rather definite levels; *e.g.*, the heart-forming areas, and the thyroid-forming area. We may inquire next to what extent differences in histogenic potency reflect detectable differences in chemical composition among the cell populations of different regions of the embryo during development. Most definitions of organ-forming areas of the early embryo, *e.g.*, the heart-forming areas, have implied that the cells of the regions of the embryo so defined are destined to become specialized at some future time for the synthesis of the specific proteins characteristic of the definitive organ; that the establishment of the heart-forming areas is a localization of prospective myosin-synthesizing (and other cardiac muscle protein-synthesizing) cells. Recent findings in our laboratory indicate that a revision of this concept may be in order for, by immunochemical methods, the progressive restriction of specific reactive groups of cardiac myosin has been demonstrated. A detailed account of the progress of the research may be found in earlier publications (Ebert, 1950, 1952, 1953). It was shown by absorption and precipitin methods that antisera produced in rabbits against extracts of adult chicken heart will react with extracts of early chick embryo prior to mor-

phogenesis of the heart. Hence, the early blastoderm must contain antigens identical with or closely related to those of adult heart. This conclusion is supported by the finding that, when early embryos are cultured in media containing antiheart serum, the development of the heart is differentially suppressed. While these findings are in themselves of considerable interest, they suggest a number of further questions. Since the antiheart sera employed are doubtless mixtures of antibodies owing to the complex nature of the antigenic fractions which elicited them, one is led to inquire as to which of the embryonic antigens are so vital to the metabolism of the embryo that combination with the specific antibodies results in suppression of differentiation and, in higher concentrations, in the death of the embryo. Our own findings constitute only one example of the cytotoxic effects of antiorgan sera. As Nace (1955) has emphasized, it may be profitable to approach the problem from the point of view that the inhibitory action of specific antibodies may be used as guides to unsuspected cellular components important to development. Yet this approach tends to suffer from operating too much in terms of complex systems. The sensitivity and specificity of the immunochemical methods suggest, however, that it might be possible to investigate the development of one or more specific proteins, to follow quantitatively the synthesis of specific proteins and investigate the effect of various conditions on the process. Recent advances in our understanding of the chemistry of muscular contraction (Szent-Györgyi, 1953; Mommaerts, 1954) enable one to pose meaningful questions concerning the synthesis of the muscle proteins. In an earlier publication (Ebert, 1953), some of these questions were stated as follows, "Do actin and myosin develop simultaneously? Is the development of each protein an independent event or does the development of one precede that of the second and have a causal role in its synthesis? Are there differences in the contractile proteins of cardiac and skeletal muscle which play a significant role in the precocious restriction of cardiac contractile proteins to the heart-forming areas of the blastoderm, and in the early onset of contractility?" In approaching the problem, an immunochemical analysis first was undertaken of the time of synthesis and distribution of myosin in the development of the heart. Questions of preparation and purity, and antigenic specificities of the myosins employed have been discussed in detail elsewhere (Ebert, 1953). It will suffice here to emphasize that, owing to marked differences in antigenic makeup of cardiac and skeletal myosins from adult chickens, it is possible to prepare, by absorption procedures, antisera highly specific for adult cardiac myosin. Such specific, absorbed antisera were employed in microprecipitin tests with extracts of whole blastoderms in prestreak through early somite stages, and of surgically isolated regions of blastoderms in mid-streak through early somite stages. The findings show clearly that cardiac myosin is synthesized in the early embryo, and can be detected in the blastoderm at the mid-streak stage (Hamburger and Hamilton stage 3); in the embryo at stage 4 (definitive primitive streak), the antigenic reactive groups of the protein are not localized, but can be detected in all sections of the embryo analyzed, being more concentrated in the middle and posterior than in the anteriormost regions. Preliminary experiments indicate that, at stages 3 and 4 at least, cardiac myosin is confined to the epiblast,

being absent from all extracts of hypoblast tested. In the embryo at stage 5 (head process) and more clearly at stage 6 (head fold), cardiac myosin is restricted in distribution to two lateral regions, in good agreement with the limits of the heart-forming areas as defined by Rawles (1943). The findings indicate that a major synthesis of cardiac myosin is initiated only after the prospective mesodermal cells have begun to move through the primitive streak. It is suggested that cardiac myosin formation is limited to the epiblast, but the evidence at hand does not permit the assignment of myosin-forming capacity more precisely. It is clear, despite the coincidence in time of the onset of myosin synthesis and cell movement through the patent streak, that cells which have not yet passed through the streak are capable of myosin formation, as shown by the widespread distribution of the protein in the stage 4 blastoderm, particularly in the most anterior levels.

The establishment of the heart-forming areas, then, clearly involves the disappearance from a large part of the embryo of an already established ability to synthesize the specific protein cardiac myosin. The information at hand does not permit an unequivocal conclusion concerning the mechanism of restriction of myosin synthesis. At least two hypotheses are possible: First, that the localization of the protein is a sorting-out or segregation phenomenon, the result of cellular movements within the mesoderm. According to this view, the over-all synthetic ability of the embryo would not be reduced, but merely aggregated in discrete regions. While this interpretation is not supported by the facts at hand concerning morphogenetic movements within the mesoderm, it cannot be ruled out, for present knowledge of morphogenetic movements does not permit a critical evaluation. According to the second hypothesis, the myosin-synthetic mechanism would be lost in the embryo except for the heart-forming regions. Barring a concomitant increased rate of synthesis in the heart-forming areas, therefore, the total synthesis of cardiac myosin should be reduced. A quantitative immunochemical analysis of the rate of accumulation of cardiac myosin in the heart-forming regions and rate of disappearance in the adjacent parts of the embryo, coupled with a radiochemical study of the rate of incorporation of labeled amino acids, as one index of rate of synthesis in the heart-forming areas, may provide the information necessary to permit a choice between these two alternatives.

Actin

It becomes of interest to examine next the development of the second major cardiac contractile protein, actin. Since the results of the investigation have not been published previously, a more detailed presentation is in order at this time. For clarity, attention must be given to questions of preparation, purity, and chemical and physical properties of actin, leading to a consideration of the question of antigenic specificity.

Method of preparation. Most of the physicochemical studies on actin have been made on preparations based on the original or modified procedures of Straub (1942, 1943). The removal of myosin from a muscle mince with ice-cold acid phosphate-KCl solution constitutes the first step of the procedure for the extraction of actin. The residue is brought to neutral pH, extracted

with n-butanol and acetone (Tsao and Bailey, 1953), and dried in air at room temperature. Actin is extracted from the dry preparation and purified by dialysis or ultracentrifugal isolation of the polymer, followed by depolymerization at pH 8.2 in the presence of ATP (Mommaerts, 1951, 1952). G-actin may be repolymerized and the process repeated until the desired purity is obtained. The extraction with butanol is recommended, for it eliminates or suppresses many of the impurities previously found in crude actin, including enzymes (Hopkins, Morgan, and Lutwak-Mann, 1938; Laki and Clark, 1951), lipids (Bailey and Perry, 1947), and coloring matter and flavoprotein (Tsao and Bailey, 1953). Butanol is particularly effective in the fission of lipoprotein complexes and in the removal of flavoprotein. The purification of actin from cardiac muscle requires repeated treatment with butanol to remove the strongly-bound flavoprotein and carotenoid components which are present in relatively large amounts. In agreement with Tsao and Bailey (1953), our spectrophotometric studies of crude actin show a sharp maximum at 3100 Å, and a weak, broad maximum near 4300 Å. The 3100-Å maximum probably rises from carotenoid material, while the 4300-Å maximum probably indicates the presence of flavoproteins. Shaking with ether or chloroform will not remove them completely.

Amino acid composition and molecular weight. The amino acid composition of actin has been determined by Feuer, Molnar, Pettko, and Straub (1948), Ivanov and Asmolova (1950), and Kominz, Hough, Symonds, and Laki, (1954). The data are summarized in TABLE 1. The recent findings of Kominz *et al.* are more meaningful than the earlier studies which antedated the purification procedures now in use.

TABLE 1
AMINO ACID COMPOSITION OF ACTIN

Amino acid	Per cent nitrogen		
	Feuer, *et al.*	Ivanov and Asmolova	Kominz, *et al.*
Cystine/2	.72	0.41–0.42	0.94
Aspartic acid	10.97	5.85	6.87
Threonine			4.95
Serine			4.70
Glutamic acid	5.49	9.2	8.45
Proline	5.08		3.69
Glycine	8.30		5.61
Alanine			5.95
Valine			3.52
Methionine	0.0	2.4–2.9	2.51
Isoleucine			4.77
Leucine			5.27
Tyrosine	1.45	2.1	2.68
Phenylalanine	0.0	trace	2.43
Histidine	2.46	4.0–4.1	4.76
Lysine	11.48	10.25	8.75
Arginine	1.60	14.2–14.6	12.75
Tryptophane	0.22	1.7–1.9	1.67
Amide NH_3			5.54

Based on the highly inaccurate tryptophane content of crude actin, Feuer *et al.*, (1948, *cf.* Szent-Györgyi, 1951) set the minimal molecular weight of actin at 70,000. This value is fortuitously nearly correct. The light-scattering method was used by Steiner, Laki, and Spicer (1952), who reported an average molecular weight of 80,000 for G-actin, the molecular weight of F-actin decreasing from 4,030,000 to 150,000 with increasing electrolyte concentration. Mommaerts (1952) obtained a still lower value of 57,000 for the monomer. Since actin possesses thixotropic properties, however, interpretations of results based on such techniques as viscosity, sedimentation, and light scattering may be open to question. Tsao (1953), using the osmotic pressure method, obtained molecular weights of 74,000, and 140,000 for actin in the monomeric and dimeric states. This finding was supported by fluorescence polarization studies based on techniques developed by G. Weber (1952). The rotational relaxation time was determined by the polarization of the fluorescence of actin molecules labeled with 1-dimethylamino-naphthalene-5-sulfonyl chloride. The relaxation time is a function of both the size and shape of the particles. By employing Simha's equation, the axial ratio can be assessed from the value of the intrinsic viscosity. Assuming a probable partial specific volume of 0.74, and 39 per cent hydration of the protein molecule, the molecular volumes and the molecular weights were derived.

The shape and dimensions of the molecule. G-actin has been described as a globular molecule on the basis of its low relative viscosity, and F-actin as a linear polymer on the basis of its high viscosity and flow birefringence. Light-scattering data support this view, but exhaustive proof is not available. Szent-Györgyi (1953) concludes that the "actin (G) globule is not only round but actually is a rather solid, hard little object, fit to act as a ball bearing . . . ."

Tsao (1953) describes the G-actin molecule as a prolate ellipsoid of revolution, with an axial ratio $\sim$ 12, the approximate dimensions for monomeric actin being therefore $\sim$ 290 Å long, and $\sim$ 24 Å wide.

G-actin molecules in solution repel each other owing to their negative electrostatic charge. These repulsive forces are eliminated by the addition of salt, which serves to suppress the negative molecular charge, permitting polymerization to occur. This development is indicated by the fact that the effectiveness of salts is a function of the properties of the cation (Mommaerts, 1952). The forces which hold the F-actin molecules together may not be very strong, as depolymerization can be readily brought about by the addition of 0.6M KI. The KI probably acts by increasing the negative charge of the particles by the absorption of I^- ions (Szent-Györgyi, 1953).

The transformation of G-actin into F-actin by the addition of salt is accompanied by a liberation of H^+ ions. When salt is added to a solution of ATP, a similar drop in pH is observed. Thus the ionization of ATP is itself affected by its binding to actin.

G-actin is not precipitated or denatured at room temperature by the addition of alcohol or acetone. The water solution can also be dried to a thin sheet in the desiccator without damage and will readily dissolve again. One of the most interesting properties of F-actin is its thixotropic nature. A concentrated solution of F-actin, when allowed to stand, will gel. Upon agitation, however,

most of the gel will pass back into solution as evidenced by a decrease in viscosity. Some will remain in the gel form, as evidenced by the persistence of birefringence and the presence of microscopic lumps, which may be seen when this solution is swirled in a flat Petri dish and observed with a polarizing microscope.

Immunochemical studies. Kesztyus, Nikodemusz, and Szilagyi (1949) reported briefly that rabbit skeletal actin is antigenic in dogs. Dog antirabbit skeletal actin serum reacted with rabbit actin in both G- and F-forms, and with F-actomyosin, but not with purified skeletal myosin. Absorption methods were not employed. These investigators reported, in addition, that rabbit skeletal actin is *isoantigenic*; *i.e.*, it elicits antibody formation in rabbits. Insufficient data are presented to enable one to evaluate critically this report. Arosio (1953) reported that rabbit actomyosin is antigenic in the guinea pig. In the present investigation, actin extracted from cardiac muscle of adult chickens has been shown to be antigenic in rabbits. Two methods have been followed in preparing purified actin; *viz.*, the ultracentrifugation technique employed by Mommaerts (1951, 1952) following extraction of crude actin according to Straub (1942, 1943), and the method of Tsao and Bailey (1953) involving butanol extraction and dialysis. A total of 11 adult male rabbits was employed. Of this number, 9 were injected with cardiac G-actin prepared by Mommaert's procedure, and 2 received cardiac G-actin prepared by the method of Tsao and Bailey. After a series of intravenous injections totaling 15 to 25 mg. of actin on alternate days over a 2-week period, followed by a 7-day rest period, each rabbit was bled. After 20 to 30 days rest, a second series of injections was started, in which the first injection of 2 to 5 mg. of protein was given intraperitoneally, followed by a total of 40 to 50 mg. of protein given intravenously in a series of injections over a two week period. Again, the rabbits were bled seven days after the last injection. The sera were sterilized by passage through a Seitz filter and stored at $-20°$ C. No preservative was added. The titer and specificity of each serum was determined by the precipitin technique. The methods were comparable to those employed in the earlier analysis of the immunochemical properties of myosin (Ebert, 1953). Altogether, 21 antisera from 11 rabbits have been assayed. Appropriate controls have been carried out with sera of uninjected rabbits. The effective serum dilution titers of the antisera tested with homologous antigen ranged from 1:32 to 1:4096. Only those sera with titers of at least 1:256 were employed in the tests to be described (12 sera). When these potent anticardiac actin sera were tested for reactivity with skeletal actin, it was found that all reacted to some extent. In parallel series of tests comparing the reactivity of anticardiac actin sera with both cardiac and skeletal actins, differences of at least 5 and generally 7 to 8 tubes were common. In further tests, antisera against cardiac actin were completely absorbed with skeletal actin, in order to remove all antibodies reactive for skeletal actin, and then tested with cardiac actin preparations. A major part of the specific activity of each serum was retained. Loss of activity due to absorption ranged from one to four tubes. In the

sections to follow, all references to anticardiac actin sera refer to these highly specific absorbed antisera.

Despite reports that the methods employed yield homogeneous actin preparations, immunochemical studies clearly show our preparations to be complex mixtures. Cardiac actin prepared by either method is a mixture of at least three types of antigenic molecules. It is encouraging to note that the cardiac actin preparations from the two extraction procedures are immunochemically closely related. Tests by the Oudin serum-agar method coupled with reciprocal absorption studies indicate that, of the three antigens present in cardiac actin, at least one is held in common with skeletal actin. It is possible, through absorption methods, to prepare antisera which are specific for the cardiac actin antigenic complex, by removing the antibody against the common antigenic component. These specific antisera serve as sensitive agents for the study of the time of appearance and distribution of cardiac actin in the chick embryo. It was demonstrated, first, that the actin (antigenic complex) present in adult heart is present in, and can be isolated from the heart of the 12-day-old chick embryo. At earlier stages, it is necessary to employ a crude extract of cardiac muscle, since a measurable yield of purified actin could not be obtained from the small quantities of material available. All preparations of cardiac muscle, both the purified actin complex and the crude extract, from embryos of 12 to 18 days, and 36 hours to 11 days, respectively, reacted positively with all antisera tested. The findings in preliminary experiments indicate that, when the specific antisera are applied directly to the embryo, *in ovo*, at 48 to 60 hours, further development of the heart is blocked, resulting in the death of the embryo. These studies demonstrate that combining groups identical or closely related to those of adult cardiac actin are present in the embryo as early as 36 hours of incubation. However, both specific antigens of the cardiac actin complex may not be present prior to the 12th day; for tests by the Oudin serum-agar method indicate only that at least one antigen is present.

This investigation has been extended to an examination of the formation and distribution of cardiac actin in the early blastoderm. The approach parallels that employed in the earlier study of the development of cardiac myosin. Extracts of intact mid-streak to early somite stage embryos and of surgically isolated regions of definitive primitive streak, head-process and head-fold stage embryos were tested for the presence of antigens of the actin-complex by sensitive precipitin methods. These experiments, in which more than 1600

Table 2

Distribution of Cardiac Actin in the Early Chick Embryo

Hamburger and Hamilton stage	Total No. of embryos extracted	No. of fresh extracts tested	No. of antisera used/extract	Total No. of test series	Section of embryo tested	Number of positive reactions	Highest titer
1	111	2	3	6	entire	0	—
2	163	3	2 or 3	8	entire	0	—
3	201	4	3 or 4	14	entire	0	—
4	356	9	2–6	25	entire	2	8

TABLE 3
DISTRIBUTION OF CARDIAC ACTIN IN THE EARLY CHICK EMBRYO

Hamburger and Hamilton stage	Total No. of embryos extracted	No. of fresh extracts tested	No. of antisera used/ extract	Total No. of test series	Section of embryo tested	Number of positive reactions	Highest titer
5	591	13	3–6	51	Entire	21/21	1024
					Anterior	0/14	—
					Middle	14/14	512
					Left	15/16	256
					Median	5/16	16
					Right	14/16	128
					Posterior	4/14	8
6–7	207	5	3–6	20	Entire	5/5	512
					Anterior	0/7	—
					Middle	7/7	512
					Left	8/8	128
					Median	1/8	4
					Right	8/8	128
					Posterior	0/7	—

early blastoderms have been analyzed in 124 test series, have established that at least one antigen of the cardiac actin complex first can be detected by precipitin methods in the embryo at the head-process stage. Further, it has been shown that when first clearly detectable in the embryo, the specific antigen is confined to those areas previously defined as the heart-forming areas. At the head-process stage, there are traces of cardiac actin in the region of the streak, but by the head-fold stage, the antigen is completely restricted in distribution. The data are presented in TABLES 2 and 3.

Discussion.

The sequence of development of the cardiac contractile proteins in the chick embryo can be summarized as follows:

(1) The major synthesis of cardiac myosin is initiated during the formation of the primitive streak; the protein first can be detected with the methods employed at the mid-streak stage.

(2) In the definitive primitive streak stage embryo, cardiac myosin is distributed throughout the epiblast; it cannot be detected in the hypoblast.

(3) Beginning at the head-process stage, cardiac myosin is restricted to the heart-forming regions of the embryo.

(4) The restriction in distribution of cardiac myosin coincides with the initiation of synthesis of at least one antigen of the cardiac actin complex which is already limited in distribution to the heart-forming areas at the time it is first detected.

At the molecular level, it would be interesting and instructive to know more in detail of possible relationships between the spatial limitation of myosin synthesis and the initial synthesis of actin. The formulation of an attack on this problem leans heavily on the kinetic analysis of cardiac myosin synthesis proposed earlier. The sequential synthesis of cardiac myosin and actin demonstrated in these experiments tends to oppose the hypothesis advanced by Kom-

inz, Hough, Symonds, and Laki (1954) that myosin may be composed of actin plus tropomyosin. This hypothesis is based largely on the fact that the amino acid compositions of actin and tropomyosin "add up" to that of myosin. Myosin has not been synthesized from mixtures of actin and tropomyosin, nor has myosin yielded these smaller proteins upon degradation.

It will be of interest next to compare our findings and interpretation with the previously unpublished findings of Johnson (1953), who has carried on an extensive independent investigation of the ontogeny of several of the major proteins of the chick heart. The following summary of the pertinent procedures and findings is presented by permission of Doctor Johnson (The Lilly Research Laboratories, Indianapolis, Ind.). The methods utilized parallel, to a considerable extent, those employed in our own laboratory. From the adult chicken heart, the following proteins were extracted, following the methods of Dubuisson (1946) and Szent-Györgyi (1951): actomyosin, (crude) actin, myosin, myogen, and globulin X. Antisera were prepared in rabbits against each of the proteins. The antisera prepared against myosin were not employed further, however, owing to the instability and possible denaturation of the antigen. Several antisera were prepared also against a sodium pyrophosphate extract of adult chicken heart and against whole chicken serum, and one antiserum was prepared against each of the following antigenic materials: saline extracts of egg white and yolk; defatted chicken serum albumin and globulin; and sodium pyrophosphate extracts of whole 72- and 96-hour embryos. A serological comparison of the several antigens was carried out by the precipitin method employing the Libby Photronreflectometer. The antigens studied fell into several major groups: antisera to actomyosin and actin each showed considerable cross reactivity with actomyosin, actin, and myosin; a second related grouping contained myogen, globulin X, and chicken serum. The pyrophosphate extract of whole heart exhibited some correspondence with all of the proteins with the possible exception of actin. In general, although some preliminary absorption studies were carried out in the determination of degrees of serological relationship between the proteins, the antisera employed as diagnostic tools were not absorbed routinely. The antisera prepared against the adult muscle and serum proteins were used as reagents to detect the time of origin of the homologous proteins. The following methods were used: (1) precipitin tests; (2) the micro-injection of antisera into the wall of the beating heart of the 72-hour embryo; (3) the addition of antisera to the embryo prior to incubation, followed by incubation for 72 hours; (4) an analysis of the development, *in vitro*, of the embryo in the presence of antisera (method of Ebert, 1950); (5) a study of the effects of antisera on the differentiation and growth of heart fragments from 72-hour embryos, cultured *in vitro*. The following findings have been selected from among many for discussion at this time. Precipitin tests indicate the following sequence of development of the proteins studied: serum proteins are present in yolk and can be detected in the blastoderm at 5 to 10 hours; myogen is detected first at 20 hours, followed by globulin X and actomyosin at 40 hours of incubation.

The latter finding is not in agreement with our own data, which clearly indicate both actin and myosin to be present and, in fact, localized to the heart-

forming regions of the embryo at the head-process stage. On the other hand, Johnson presents data in support of our conclusion, namely that embryos grown in the presence of antiactomyosin sera, *in ovo* and *in vitro* are abnormal, in that many lack hearts completely or have malformed hearts. Nonspecific effects were noted in addition, a fact which is not surprising since critical absorption procedures were not carried out, and, as noted earlier, there is a considerable degree of cross reaction between antisera to cardiac actin and myosin and the corresponding skeletal proteins. In addition to the effects on heart morphogenesis, antiactomyosin sera inhibited general embryonic growth *in ovo* and inhibited somite formation *in vitro*. The divergent findings from the precipitin technique and the *in ovo* and *in vitro* effects of antiactomyosin sera may be interpreted simply as demonstrating that the culture methods are more subtle tests than the precipitin techniques employed. Johnson proposes a more complex interpretation: while the effects of antiactomyosin sera are due to combination of the antiserum with some material essential to the formation of the heart, that material is an "incomplete" antigen, since it does not react with the antiserum in precipitin tests. He argues further that such a molecule might best fit the category of a "precursor" molcule. Since there is a beating heart prior to 40 hours, Johnson proposes that pulsation is due to a functional precursor of myosin, "myosinogen." He speculates that myosinogen is converted catalytically (by enzymatic action of the previously formed myogen) to myosin plus an inert protein, globulin X. In view of our findings employing absorbed antisera produced against more purified proteins in sensitive microprecipitin methods, this hypothesis appears unnecessary. Once again, however, these findings re-emphasize that, whenever possible, both immunochemical and biological methods must be employed in studies of the time of synthesis and distribution of tissue-specific proteins.

Recent studies by Gergely (1953) and Mihalyi and A. G. Szent-Györgyi (1953 a, b, c) have demonstrated that by treatment with trypsin, myosin can be split into two relatively well-defined products, L- and H-meromyosin. The former product resembles myosin in its solubility characteristics and can be crystallized. The latter is soluble at low salt concentrations but carries the adenosinetriphosphatase (ATPase) activity and actin-combining capacity. In view of these findings, it should be of interest to determine the time of development and distribution of ATPase activity in the heart-forming regions. To date, ATPase has not been studied in the early stages of cardiogenesis, nor in fact in the entire chick embryo earlier than three days of incubation. Cafiero (1950) has studied the ATPase activity of the entire embryo beginning on the third day of incubation; Robinson (1952) suggests from a study of embryos at later stages that ATPase activity increases progressively in the myofibrillar fraction, suggesting a gradual association of the myofibril and ATPase. Moog and Steinbach (1945) have studied the apyrase activity of the entire embryo. Again the period prior to three days has not been considered. The development of standardized microtechniques for fibrillar (calcium-activated) and sarcosomal (magnesium-activated) ATPases would constitute an important technical advance in this direction.

The Metabolic Basis of Cardiogenesis

Recent electron microscope studies (Porter and Thompson, 1947; Oberling, Bernhard, Guerin, and Harel, 1950; Selby and Berger, 1952; Porter, 1954, a, b) have shown that the cytoplasm of growing cells contains numerous dense bodies, 25 to 250 mμ in diameter, which, in terms of showing paired associations and size gradations, suggest that they may be dividing and eventually transforming into fundamental elements of the cytoplasm, *e.g.*, mitochondria and vesiculated endoplasmic reticulum. Porter has suggested, as a working hypothesis, that these "growth granules" are centers of formation of cytoplasmic inclusions of special form. According to Porter (1954a), developing myoblasts of *Amblystoma punctatum* contain three prominent components: myofibrils, ground cytoplasm (sarcoplasm), and a number of slender bodies of varying lengths and widths, essentially the same size as the growth granules. These particulates are often closely associated with the myofibrils. There is no evidence of a direct transformation of the granules into myofibrils. There is a suggestion, however, that the condensation or polymerization of the myofibrils out of the matrix occurs in the vicinity of the granules. The relationship of these granules to the definitive heart muscle mitochondria is not clear. Although the early literature contains several reports of mitochondrial participation in myofibril formation (Benda, 1899; Duesberg, 1909), no critical experimental evidence is available concerning the interrelationships of mitochondrial differentiation and myofibril formation. Goss (1932, 1933, 1938, 1940) was unable to observe the transformation of mitochondria into fibrils, although he did find that an increase in number and size of cardiac mitochondria is the first among the cytological changes observed. Cross-striations appear to be due in part to the sarcosomes (Goss, 1932, 1933; Harman and Feigelson, 1952; Weinstein, 1954). Goss concluded that mitochondria do not play a direct role in the formation of myofibrils. He suggested that the differentiation of mitochondria might be linked with the growing intensity and effectiveness of contraction. This suggestion is of particular interest when considered in the light of Sippel's (1954) study of the growth of succinoxidase activity in the hearts of chick and rat embryos. Succinoxidase, of course, is located almost exclusively on the mitochondria in adult tissues (Schneider and Hogeboom, 1951). Let us consider the chick as illustrative of the findings: succinoxidase activity, measured on ventricle homogenates using manometric techniques, increases in two cycles during development. The first rapid increase occurs by 48 hours in the chick and continues for about three days. A plateau in the succinoxidase growth curve follows, lasting three to four days, succeeded by another increase in activity to the termination of embryonic development. However, in the period from 24 to 48 hours of development, during which the heart is formed and begins to function, there is little change in the succinoxidase activity of the heart-forming tissues.

The first contractions (which occur in the ventricle in all the forms critically studied as yet) are nonpropagating local twitchings involving only a few cells. It may be helpful to consider the gradual extension of contractile activity and initiation of regular rhythmic contraction as linked to the more intimate

association of mitochondria and myofibrils. Sippel suggests, in addition, that, during this period, the mitochondria may differentiate in the sense that succinoxidase and other enzymes may migrate from the general cytoplasm to the mitochondria. Critical evidence on this point is almost entirely lacking. It is encouraging to note Sippel's emphasis on the fact that mitochondria (and other particulates) undergo differentiation. All too frequently, hypotheses concerning developmental mechanisms revolve about the interactions of particulates, based on the activities of the particulates in the adult, assuming that the particulates, *per se*, are stable throughout ontogeny. Immunochemical studies of the differentiation of organ-specific properties of mitochondria have been initiated in our laboratory. A number of technical difficulties must be surmounted (*cf.* Furth and Kabat, 1941; Dulaney, Goldsmith, Arnesen and Buxton, 1949; Malmgren and Bennison, 1950; MacFarlane and Datta, 1954) before the effectiveness of this tool can be evaluated critically.

Except for the investigation being carried on by Sippel, relatively little attention has been paid recently to the metabolism of the heart-forming regions. Perhaps the most fruitful lead for further analysis comes from the study by Spratt (1950) of the effects of metabolic inhibitors on the differentiation, *in vitro*, of the early chick blastoderm. Embryos explanted to glucose media containing either 10^{-4} to 5×10^{-5} M monoiodoacetate or 10^{-2} M fluoride degenerate rapidly. At lower concentrations of iodoacetate (2×10^{-5} M), the central nervous system degenerates or fails to form, but the heart develops and pulsates. Fluoride has an opposite effect, for concentrations which cause degeneration of the heart (5×10^{-3} M) have no appreciable effect on the development of the nervous system. Other inhibitors: namely, citrate, malonate, and cyanide result in effects similar to those produced by iodoacetate. The observation that mesodermal derivatives are more sensitive to fluoride than ectodermal derivatives, and that ectodermal derivatives are more sensitive to monoiodoacetate, is interpreted by Spratt as indicating differences in the metabolic processes underlying the formation and maintenance of the two germ layer derivatives (the brain is also more sensitive to oxygen deficiency than is the heart). Spratt offers, as a working hypothesis, that the brain depends primarily on oxidative metabolism, the heart upon (anaerobic) glycolysis. The finding that fluoride affects the development of the heart presents an attractive target for further analysis. Among the questions raised are the following: At what level is the development of the heart blocked? Is the synthesis of the cardiac proteins affected? Are morphogenetic movements inhibited? Here the problem rests unsolved. One might profitably consider the inhibitor as acting at the mitochondrial enzyme level, resulting in the failure of the formed myofibrils to become organized as an efficient contractile system. Such an investigation, which should elucidate further the relationship between mitochondrial differentiation and the functional differentiation of the contractile system, is in progress.

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EVENTS ASSOCIATED WITH THE DEVELOPMENT OF NERVE AND HEPATIC CELLS*

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The studies reported in this paper were undertaken primarily to gain insight into changes which occur in the nerve cells of the frontal cerebral cortex during their development. The approach has depended upon techniques and concepts of histology, physiology, and biochemistry on the thesis that the results of one approach will be illuminated by those from another. A less extensive examination of the developing liver has been followed as a step towards determining whether certain biochemical changes found in nerve cells are unique or whether they are paralleled by changes occurring during the maturation of other organs.

Our observations on the cerebral cortex have been largely confined to the frontal cortex of the guinea pig. The cells of the cortex are derived from the matrix layer lying next to the ventricle. Neuroblasts and spongioblasts then migrate through an intermediate zone of relatively small population to augment those already present in the cortical layer. Our first essential step was to establish the time during development at which the primitive neuroblast assumes the cytological characteristics of a cell which is clearly more mature and which may be called a neuron. Attention has been given primarily to three cytological changes which occur during the development of cortical nerve cells: the appearance of cell processes; the appearance of the typical cellular inclusion; Nissl bodies; and the growth and maturation of the nucleus.

These microscopic studies were made by V. B. Peters,[1] whose first aim was to determine how early in development definitive processes of the nerve cells of the frontal cortex make their appearance. The guinea pig has a gestation period of about 66 days. At the 41st day, about two thirds of the way through pregnancy, only a few of the nerve cells have definitive processes. At the 45th day, long processes are evident in most of the cells. When the whole depth of the cortex is viewed under the phase-contrast microscope, the change is a very dramatic one. The cells next were examined for clumps of basophilic material, the Nissl bodies. Here, again, the 41st to the 45th days of gestation are critical ones. Before the 41st day, Nissl substance is absent; at about the 41st day, it appears for the first time and then rapidly accumulates during the next three days, the percentage of cells containing it at the 44th day being only slightly less than in the adult. Finally, using the ingenious methods of Chalkley[2, 3] for quantitative morphologic analysis as well as direct measurement, the average volume of the nuclei of the nerve cells was estimated during development. These measurements gave the result that the nucleus apparently ceases to increase in volume at the critical stage of 41 to 45 days (FIGURE 1). This finding suggests the possibility that the nucleus reaches a considerable degree of maturity before the onset of fundamental changes in the cytoplasm evi-

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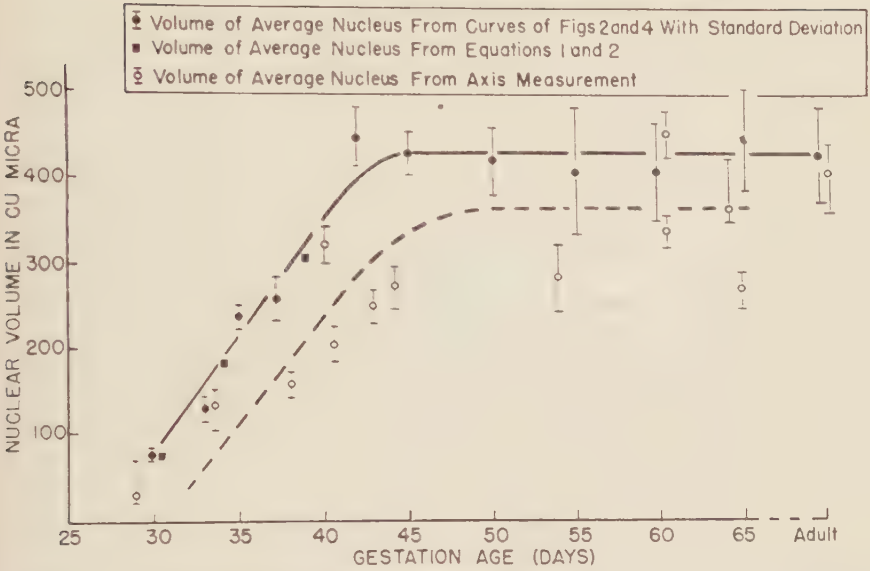

FIGURE 1. Change in volume of the average nucleus with gestation age. Volume from measurement of nuclear axes has been calculated for a prolate spheroid (smallest value), sphere (intermediate value), and oblate spheroid (largest value), and is so indicated. The broken line has been drawn by inspection to approximate these measurements. The solid line has been drawn to fit the measurements obtained with Chalkley's methods. (From the original of figure 5. V. B. Peters & L. B. Flexner. 1950. Am. J. Anat. **86:** 148.)

denced by the rapid accumulation of Nissl bodies and the intense elaboration of processes.

Additional evidence for nuclear maturity at this time comes from observations on the nucleolus. LaVelle[4] has studied the same area of the fetal cortex of the guinea pig as we have. Using the Feulgen technique and buffered thionin, he found the series of changes shown in FIGURE 2. The black areas in the diagram of the nucleolus are those which stain intensely with thionin or the Feulgen reagent. The white areas stain feebly or not at all. There is a progressive change in the Feulgen-positive material from the homogenous distribution found in the young fetus to the vacuolated nucleolus, with a thin shell of desoxypentose nucleic acid in the fetus at 45 days and in the adult. Again, the most striking change occurs between the 40th and 45th days and recalls

FIGURE 2. Drawings of nucleolar configurations which typically appear when stained by the Feulgen technique. The relative increase in nucleolar size with age is not indicated. (From the original of figure 1. A. LaVelle. 1951. J. Comp. Neurol. **94:** 457.)

the well-known theory of Caspersson[5] and Hydén[6] on the relationship of the nucleolus to the synthesis of protein.

As has been said, these cytological changes which have been described may be taken as evidence of the rather sudden differentiation of a relatively immature cell, the neuroblast, into a cell with more mature characteristics, the neuron. Are these morphological changes accompanied by demonstrable biochemical changes? Much of the biochemical observation on differentiation is concerned with the activities of enzymes. The enzyme apyrase or ATPase (FIGURE 3) has a relatively low and constant level of activity for the 14 days preceding the 42nd day of gestation, when it begins sharply to increase towards its threefold greater activity in the adult.[7] Changes of the same kind have been found in the activities of respiratory enzymes.[8] Thus, succinic dehydrogenase follows much the same pattern as apyrase. The dehydrogenase has a relatively low and constant level of activity up to about the 41st day, then begins to increase to the twofold or threefold greater activity of the adult. Cytochrome oxidase also begins to increase at much the same time (FIGURE 4) and, at term, reaches the activity of the adult, an over-all increase of fivefold.

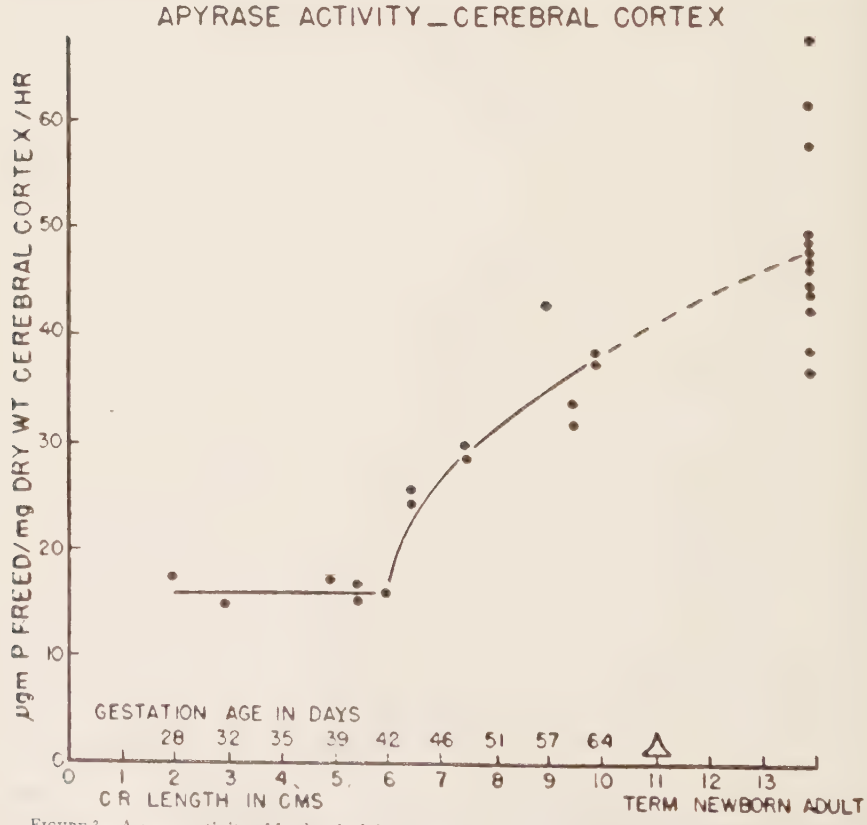

FIGURE 3. Apyrase activity of fetal and adult cerebral cortex. (From the original of figure 1. J. B. Flexner & L. B. Flexner. 1948. J. Cell. Comp. Physiol. **31**: 315.)

Observations, as yet tentative and incomplete, suggest that the glycolytic enzyme, aldolase, responsible for the conversion of fructose-1,6-diphosphate to triose phosphate increases fivefold from the critical period to term.[9]

The prime source of energy for the cerebral cortex, as for other parts of the nervous system, is glucose. Certain predictions are possible, if the measured activities of glycolytic and respiratory enzymes have functional meaning and if they are typical of those enzymes involved in rate-limiting reactions. Increase in activity of enzymes of glycolysis may be expected to lead to an increase in rate of utilization of glucose. Increase in respiratory enzymes may be accompanied by increase in rate of production of useful energy per mole of glucose, which disappears. We are presently investigating this problem and, although our observations are not complete, those that have been made substantiate these predictions.[11] The investigations have been made *in vitro* using, as substrate, glucose uniformly labelled with C^{14}. That glucose is the

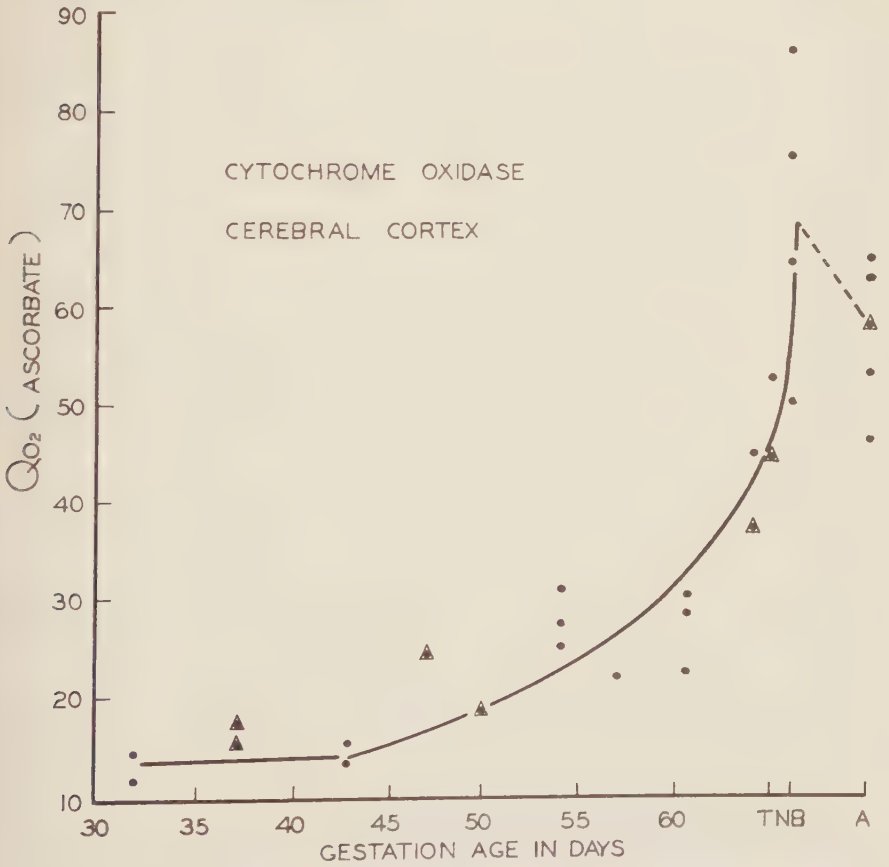

FIGURE 4. Cytochrome oxidase activity of fetal and adult frontal cerebral cortex. Results with the method of Schneider and Potter[19] are expressed in terms of cubic millimeters of oxygen consumed per milligram wet weight of cell phase per hour and are indicated by circles. Check results with a spectrophotometric method are indicated by triangles.

sole or preponderant substrate being oxidized under these circumstances is shown by the reasonably close correspondence of the specific activity of the CO_2 produced with that of the glucose serving as substrate. That rate of glucose utilization is increased with maturation of the nerve cells is evident from these examples, typical of our experience to date in phosphate buffer. In the cortex of the precritical stage containing neuroblasts, 0.7 micromoles of glucose are utilized per 100 mg. wet weight per hour. In the postcritical stage, with nerve cells rapidly synthesizing neuroplasm, the value is 1.2 micromoles while, in the adult, it is 2.1 micromoles. Rate of glucose oxidation again correlates well, qualitatively, with the measurement of enzyme activities as exemplified by the following findings: 0.3 micromoles of glucose oxidized per hour per 100 mg. wet weight of cortex of the precritical stage, 0.6 micromoles in the postcritical stage, and 1.3 micromoles in the adult. The over-all conclusion is that both glycolytic and aerobic mechanisms become more effective with differentiation of the neuroblast into the nerve cell and consequently make available to the nerve cell, as it matures, an increasing amount of useful energy from glucose.

It might be supposed from the results with the preceding enzymes that all enzymes begin to increase in activity at a common critical period and, indeed,

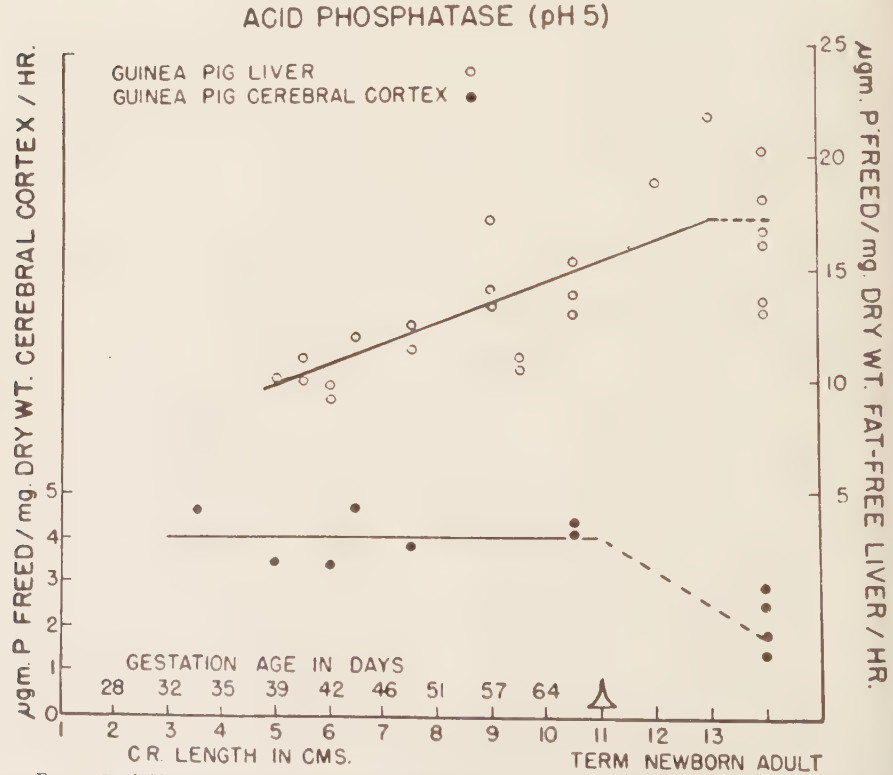

FIGURE 5. Acid phosphatase activities of fetal and adult cerebral cortex and liver. (From the original of figure 3. J. B. Flexner & L. B. Flexner. 1948. J. Cell. Comp. Physiol. **31**: 317.)

that all are at a lower activity in the fetus than in the adult. That this is not the case is shown by the results with acid phosphatase and cholinesterase. On a dry weight basis, acid phosphatase (FIGURE 5) appears to be at a rather constant level of activity in the cortex of the fetal guinea pig before birth and, indeed, to have a higher activity in the fetal cortex than in that of the adult.[7] Kavaler and Kimel[12] found a small but detectable level of cholinesterase activity as early as the 28th day of gestation in the frontal cerebral cortex of the guinea pig (FIGURE 6). This activity remains fairly constant until the 35th day, when cholinesterase rapidly begins to increase to reach an activity, at term, only a little less than that in the adult, an over-all increase of 10-fold. Kavaler and Kimel[12] point out that the initial rise in cholinesterase activity

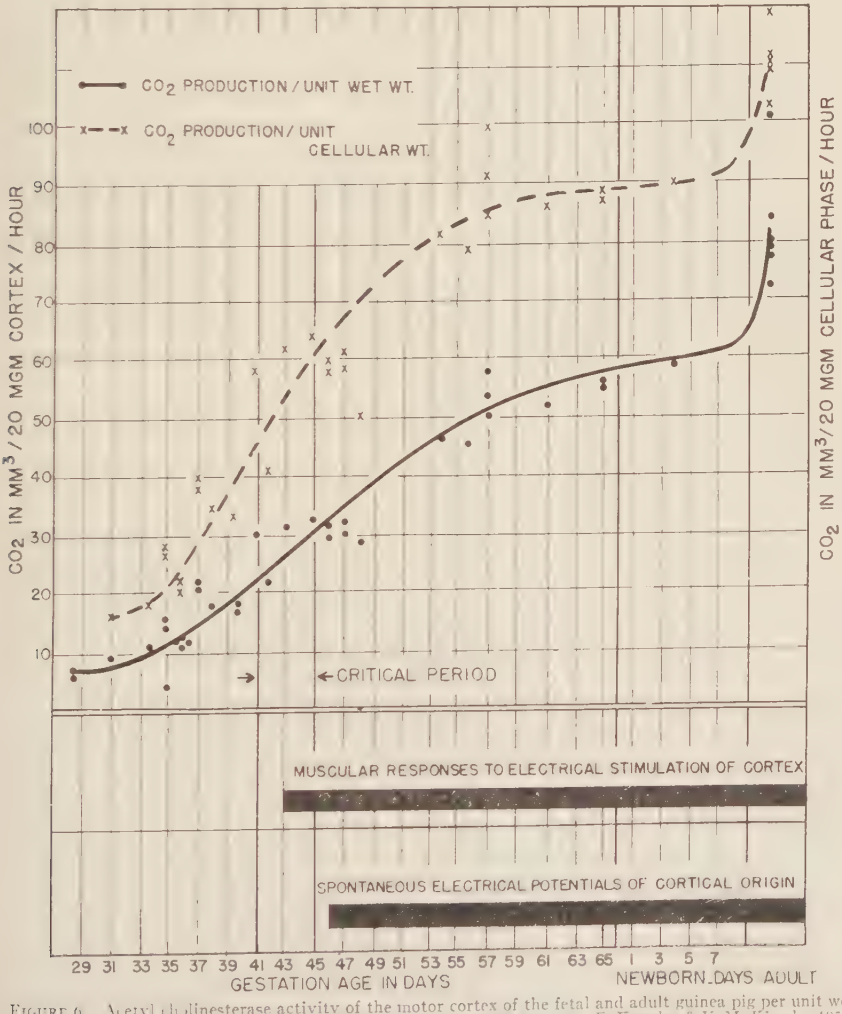

FIGURE 6. Acetylcholinesterase activity of the motor cortex of the fetal and adult guinea pig per unit wet weight and per unit weight of cellular phase. [From the original of figure 1. F. Kavaler & V. M. Kimel 1952. J. Comp. Neurol. **96:** 113.)

precedes, by six days, the beginning of the period critical for cytological differentiation of nerve cells. On the basis of the evidence that glial cells contain cholinesterase, they suggest that this early initial rise in cholinesterase activity, at the 35th day, may be an indication of biochemical differentiation of glial cells.

Interesting results have been obtained in an effort to measure succinoxidase activity.[13] Succinoxidase activity depends upon an enzyme chain which includes succinic dehydrogenase, cytochrome *c* and cytochrome oxidase. Our experience with the fetal guinea pig was much like that with the fetal pig. At early stages of gestation, the increment of oxygen consumption on addition of succinate to minced cortex, that is, the succinoxidase activity, was so small as to be uncertain (FIGURE 7). This low activity may be caused by a very low concentration of cytochrome *c*, or it may be due to other factors, among them failure of the succinate to penetrate the cell wall. To test this last possibility, the response was measured of a concentrated homogenate (in which the cell membranes are disrupted) to the addition of succinate. As shown in FIGURE 7, this response was definitive and considerably greater than with the minced tissue. Minced tissue continued to give a clearly lower re-

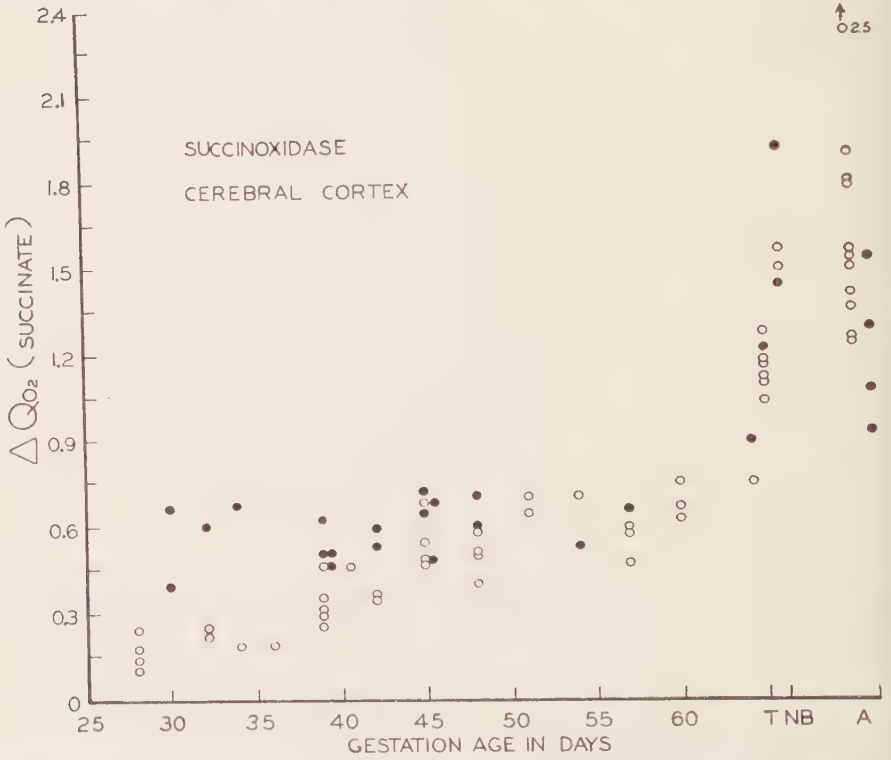

FIGURE 7. Succinoxidase activity of minced and homogenized fetal and adult frontal cerebral cortex. Results are expressed in terms of the increment, on addition of succinate, in cubic millimeters of oxygen consumed per milligram wet weight per hour. Solid circles = homogenized cortex; open circles = minced cortex. (From the original of figure 3. L. B. Flexner, E. L. Belknap, Jr., & J. B. Flexner. 1953. J. Cell. Comp. Physiol. **42**: 151.)

sponse than homogenates up to the 45th day. This behavior is explained by assuming that the cell membrane, at early stages of development, is relatively impermeable to succinate, and that maturation is accompanied by an increase in permeability.

What can be said of the beginnings of function in the nerve cells of the frontal cerebral cortex? That is, can the onset of function be correlated with the structural and chemical changes which have just been described? This problem has been approached in three ways: first, by observing peripheral muscular response to electrical stimulation of the cortex; second, by recoding the electrical activity of the cortex; and, third, by observing the reflex behavior of the fetus.

Kimel and Kavaler,[14] in their stimulation experiments on the fetal cortex, substantiated the observations of Carmichael[15] on reflex activity and found, as he did, that reflex movements of the foreleg and neck can be elicited on the 31st day of gestation; of the fingers and toes on the 35th day; and of the jaw on the 38th day. These observations demonstrated that their results on electrical stimulation of the cerebral cortex are referable to change in the cortical neurons themselves, since the other parts of the motor system (internuncial neurons, anterior horn cells, and motor end plates) have become mature before cortical stimulation is effective. FIGURE 8 shows the location of the motor areas on the surface of the cortex of the adult guinea pig. The areas which Peters and Flexner[1] studied cytologically are located in the anterior two thirds of the frontal cortex; *i.e.*, those areas concerned with movements of neck, upper and lower lip, spreading of the fingers, and mastication. As

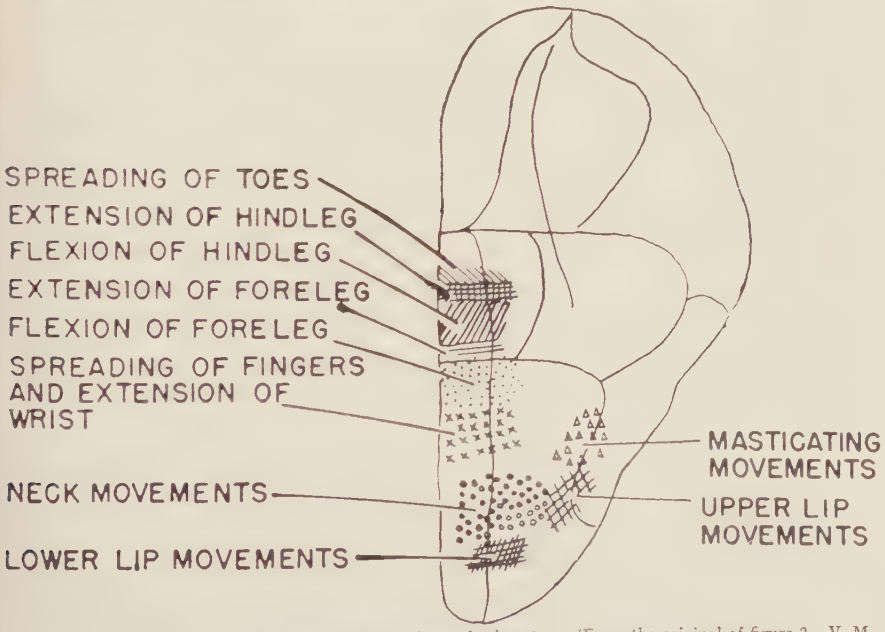

FIGURE 8. Location of motor areas on guinea pig cerebral cortex. (From the original of figure 2. V. M. Kimel & F. Kavaler. 1951. J. Comp. Neurol. **94:** 260.)

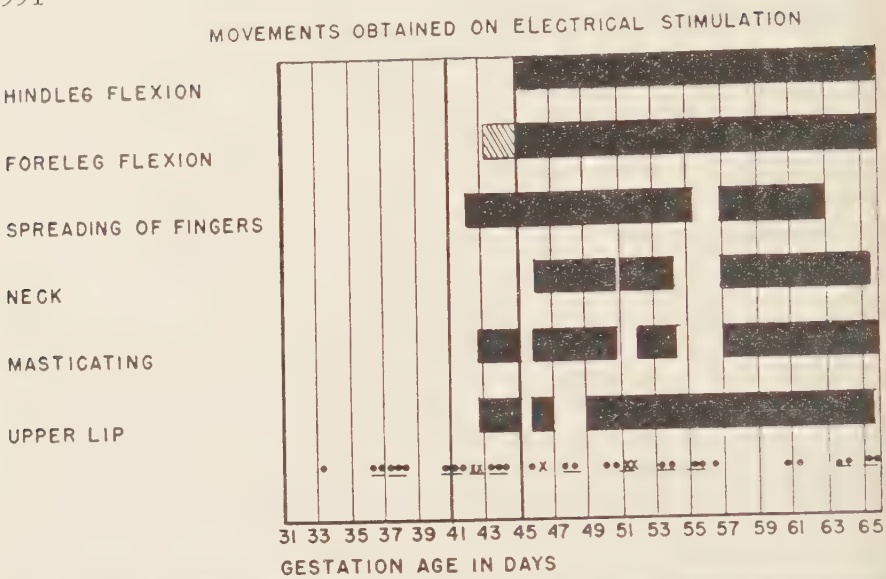

FIGURE 9. Dots = fetuses whose ages were determined from crown-rump length. Estimated error is plus or minus two days. Crosses = dated pregnancies. Error is plus or minus one day. Lines below dots and crosses indicate littermates. The black bar indicates the presence of a given response listed on the left. Blank spaces in the bar indicate that the response was not obtained from the preparation studied at that particular stage in gestation (gestation ages shown at the bottom). The shaded area indicates a partial, but typical, response. (From the original of figure 3. V. M. Kimel & F. Kavaler. 1951. J. Comp. Neurol. **94:** 261.)

shown in FIGURE 9, these movements were first elicited by cortical stimulation on the 42nd to the 46th days, at or about the time found to be critical for cytological and biochemical differentiation.

Jasper, Bridgman, and Carmichael[16] first recorded rhythmic electrical potentials from the cortex of the fetal guinea pig at about the end of the period critical for cytological differentiation and for chemical changes. Flexner, Tyler, and Gallant[17] have confirmed these results and have demonstrated that these electrical potentials arise, at least in part, from the cortex itself and not wholly from the underlying brain. In these experiments, the spinal cords of pregnant animals were transected so the fetuses could be studied in the absence of anesthesia. The fetuses were then delivered into a saline bath at body temperature with caution to maintain the placental circulation. As suggested by Jasper, Bridgman, and Carmichael, to test for artefact, the umbilical cord was clamped. In the absence of artefact, the potential changes promptly disappeared as a consequence of anoxia produced by clamping the cord. The cortical origin of the potentials was tested for by applying strychnine to the surface of the cortex and observing the prompt appearance of strychnine spikes. Potential changes were clearly evident, in agreement with Jasper, Bridgman, and Carmichael, in a fetus at the end of the critical period, 46 days of gestation (FIGURE 10). After application of strychnine, there were typical spikes of activity. When the umbilical cord was clamped, all activity except a small artefact disappeared. By contrast, on the 44th day of gestation no spontaneous activity could be recorded, and application of strychnine was without effect. Consequently, the

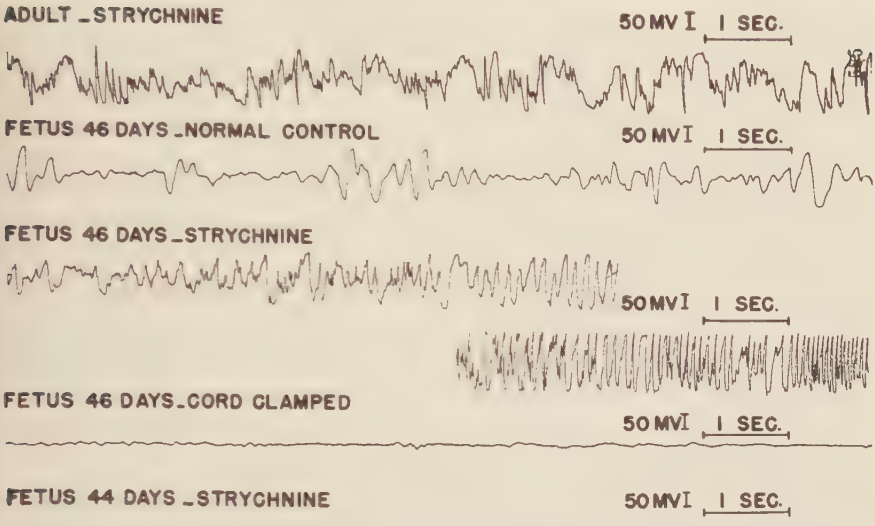

FIGURE 10. *Line* 1 (top): Individual spikes and short bursts of spikes recorded from cortex of young adult guinea pig after topical application of strychnine. *Line* 2: Rhythmic electrical activity recorded from cortex of 46-day fetus. In this example, the basic underlying frequency varies from five to eight per second. *Line* 3 (broken line): First section shows individual spikes recorded from cortex of 46-day fetus after topical application of strychnine. Second section demonstrates bursts of strychnine spikes from same preparation. Underlying the spikes is a basic five to eight per second wave pattern. *Line* 4: An almost isoelectric line recorded from the cortex of 46-day fetus within 40 seconds of clamping the umbilical cord. *Lines* 2, 3, and 4 are from the same preparation, and are typical of the findings from the critical stage up to birth. *Line* 5: Isoelectric line recorded from cortex of 44-day fetus (precritical stage) after topical application of strychnine. (From the original of figure 1. L. B. Flexner *et al.* 1950. J. Neurophysiol. **13:** 429.)

evidence indicates that rhythmic electrical activity can first be recorded from the frontal cerebral cortex at the end of the period critical for morphological and certain biochemical changes, and that this activity arises, at least in part, from cortical cells themselves.

The appearance of brain potentials and of a positive response to cortical stimulation can be related to indications of the onset of function in higher parts of the central nervous system revealed by changes in the behavior of the fetal guinea pig, as observed by Carmichael.[15] The mature cortex apparently exerts a dominant suppressor or inhibitory effect on reflex response to external stimulation. Carmichael found that the activity exhibited in reflex response in younger fetuses is much greater than in older. The decrease in response occurs at about the same time, as does the appearance of cortical electrical activity, indicating once again that function begins in the cortex of the guinea pig around the 46th day. Consistent with this observation, Carmichael has found that high transection of the spinal cord causes a marked increase in reflex response at about the same time that rhythmic electrical activity first appears.

An observation has been made which may give some insight into an event responsible for the onset of spontaneous electrical activity.[18] Hodgkin and Katz,[19] as well as other investigators, now have evidence that the action potential of nerve may be dependent upon the sudden development of a selective permeability to sodium ions. There is evidence that much the same thing may happen in the cortex when it first becomes electrically active. The dem-

onstration depends upon the concept that the bulk of the chloride of the cortex is extracellular, and that the cells themselves are relatively free of chloride.[20] The extracellular space, consequently, can be estimated from the ratio of the concentration of chloride in the cortex to that in the blood plasma.

In FIGURE 11, the results of estimation of the extracellular phase with chloride are shown by the solid circles and the curve fitted to them. Enough is known of the quantitative aspects of histogenesis of the cortex and, particularly, of the volume of the cortex occupied by nerve cell bodies to permit some interpretation of the observed changes in the apparent weight of the extracellular phase during gestation. From the 33rd to the 41st days, when the extracellular phase increases, the neuroblasts become more widely spaced, so that, in spite of increase in size of the individual cell, the percentage of cortex occupied by nerve cell bodies diminishes.[1] During this period, there is the indicated increase in volume of the extracellular phase. After a gestation age of 45 days, there is little change in the volume of cortex occupied by nerve cell bodies, but cell processes increase greatly in size and number. This increase is reflected in diminution in size of the extracellular compartment and in a corresponding increase in the cellular phase.

As shown by the open circles of FIGURE 11, the volume of tissue in which radiosodium is distributed is apparently the same as that of chloride, up to 40 days of gestation. At 46 days, however, and thereafter, sodium is apparently

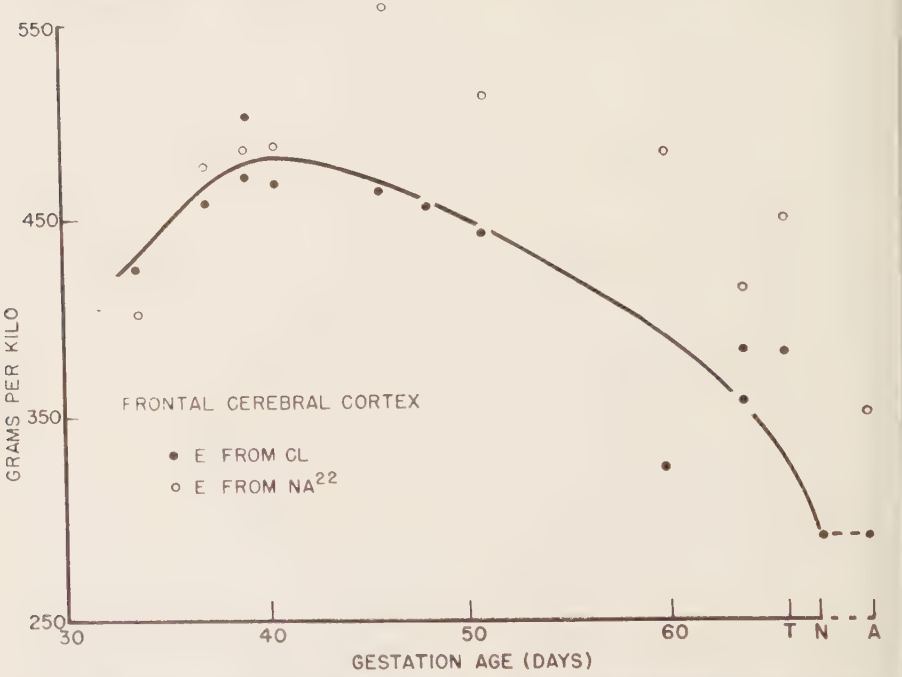

FIGURE 11. Weight in grams of the extracellular phase per kilogram of frontal cerebral cortex as calculated from the concentration of chloride and radiosodium in tissue and blood serum. The discrepancy between the two sets of values after the 45th day suggests that the neurons become permeable to sodium at this time. (From the original of figure 1. L. B. Flexner & J. B. Flexner. 1949. J. Cell. Comp. Physiol. **34:** 119.)

distributed in a considerably larger volume than chloride. This increase may be tentatively supposed to mean that nerve cells at this time become permeable to sodium and, since this time is the same as that at which electrical activity appears, it is possible that increase in permeability to sodium may be causally related to the appearance of electrical activity, in conformity with the thesis of Hodgkin and Katz.[14] The suggestion that maturation of the nerve cell is accompanied by an increase in its permeability to sodium gains some support from the apparent change in permeability to succinate previously noted. It must be emphasized, however, that this interpretation is to be regarded as tentative, and not without at least one attractive and forceful alternative. As pointed out by Lowry,[2] the apparent expansion of the sodium space may be accounted for to an unknown degree by the combination of sodium with acidic lipids, which increase at the end of the critical period.[22] It is possible, of course, that both increase in permeability and combination with lipid affect the distribution of sodium at the end of the critical period.

For the sake of uniformity, I shall restrict what I have to say about the developing liver to observations which parallel those made upon the cerebral cortex, and therefore I shall speak only of enzyme activities, omitting other studies which we have made.[15, 22-25] The principal question is whether the liver, during that part of gestation which we have studied, shows changes of the same kind as the cerebral cortex and so suggests a common pattern of en-

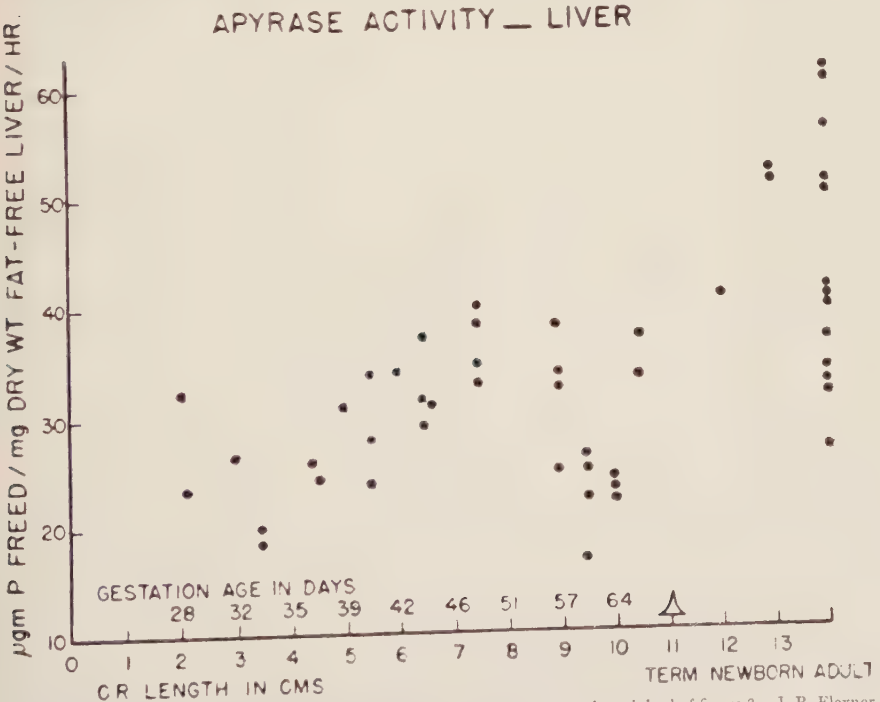

FIGURE 12. Apyrase activity of fetal and adult liver. (Modified from the original of figure 2. J. B. Flexner & L. B. Flexner. 1948. J. Cell. Comp. Physiol. **31**: 311.)

zyme change which accompanies maturation. Some suggestion that this pattern might be true comes from older studies on chorioid plexus[26] and kidney,[27] where functional maturation of the plexus and of the nephron, as in the cerebral cortex, has been demonstrated to be accompanied by abrupt increase in activity of the cytochrome-cytochrome oxidase system. It should be pointed out that interpretation of the results with the liver is often made difficult by their irregularity and by the fact that the liver is a hematopoietic organ during much of gestation.

It will be remembered that, in the cerebral cortex, the pattern of activity of some enzymes is characterized by the beginnings of an increase at a common critical period. This pattern was observed for ATPase, cytochrome oxidase, and succinic dehydrogenase. ATPase in the fetal liver, as shown in FIGURE 12, is of irregular activity and shows no clear trend up to term.[7] Succinic dehydrogenase (FIGURE 13), again with considerable variation in its activity, shows no clear change up to term, although there is a suggestion of an increase in activity at or shortly after birth.[8] Cytochrome oxidase (FIGURE 14) increases in

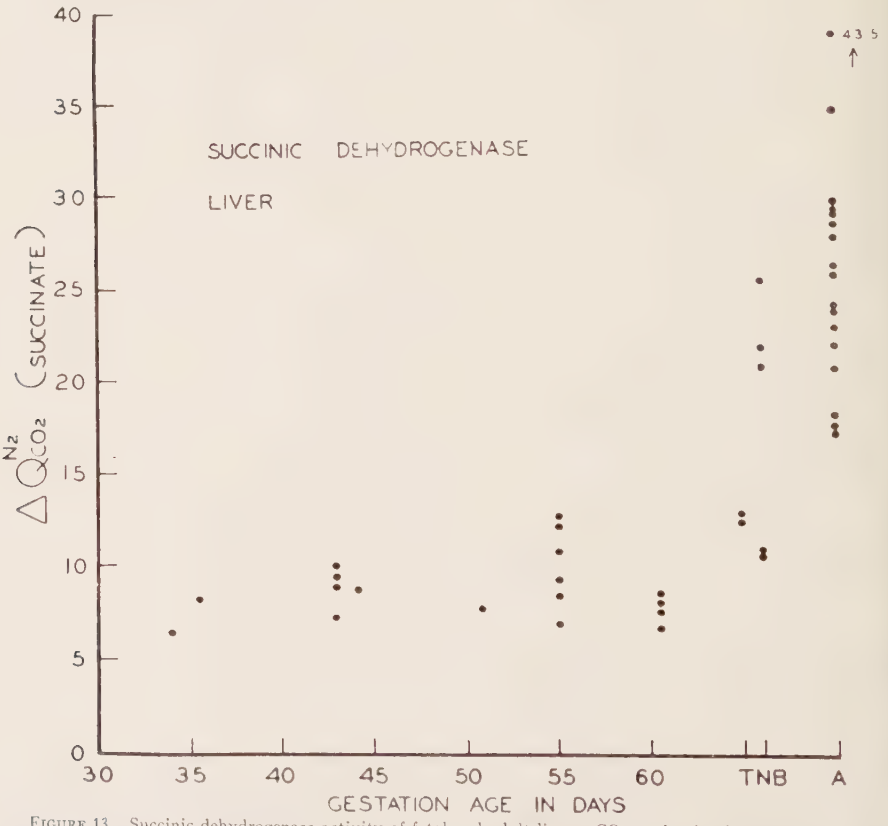

FIGURE 13. Succinic dehydrogenase activity of fetal and adult liver. CO_2 production is in terms of cubic millimeters CO_2 per milligram wet weight of cells (fat and glycogen free) per hour, as observed with method utilizing homogenized tissue and ferricyanide. T = term, NB = newborn, A = adult. (Modified from the original of figure 5 L. B. Flexner, E. L. Belknap, Jr., & J. B. Flexner 1953. J. Cell. Comp. Physiol. **42**: 151.)

activity from the 32nd day to term, but without evidence of a critical period, which, however, cannot be excluded as being present before the 32nd day.[8] Acid phosphatase in the cerebral cortex is at a relatively constant level throughout gestation, and higher in the fetus than in the adult. By contrast,[8] this enzyme appears to increase in activity to term in the liver, but without a critical period (FIGURE 5). The only enzyme known to me for which a critical period has been clearly demonstrated in the liver of the guinea pig is glucose-6-phosphatase, concerned in the conversion of glycogen to glucose. Nemeth[28] has shown that glucose-6-phosphatase is absent until the 64th day of gestation, when it rapidly begins to increase to its maximal activity observed in the newborn animal three to five days later (FIGURE 15). So far as can be judged from the observations at hand, consequently, it appears that enzyme activities of cerebral cortex and liver do not follow a common pattern during development but are distinctive, with relation to one another. The only similarities are to be found in the over-all increase in activity of cytochrome oxidase and succinic dehydrogenase, which occur with maturation.

By way of summary, let me fit the observations which I have presented into

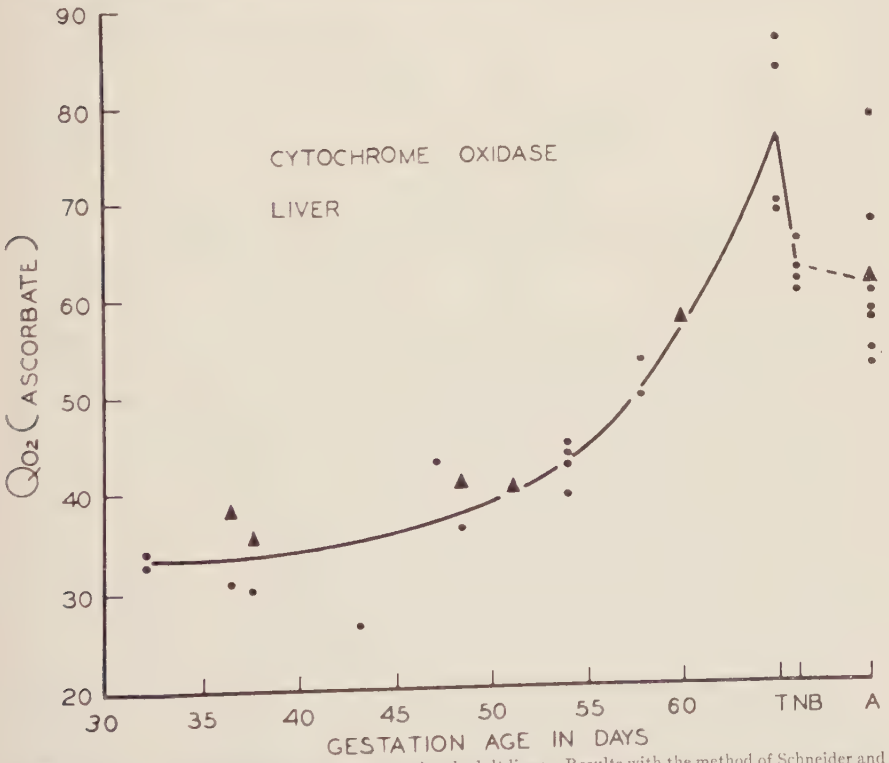

FIGURE 14. Cytochrome oxidase activity of fetal and adult liver. Results with the method of Schneider and Potter[10] are expressed in terms of cubic millimeters of oxygen consumed per milligram wet weight of cell phase (fat and glycogen free) per hour and are indicated by circles. Check results with the spectrophotometric method are indicated by triangles and have been plotted by placing the adult value at the average Q_{O_2} (ascorbate) of the adult. (From the original of figure 4. L. B. Flexner, E. L. Belknap, Jr., & I. B. Flexner. 1953. J. Cell. Comp. Physiol. **42**: 151.)

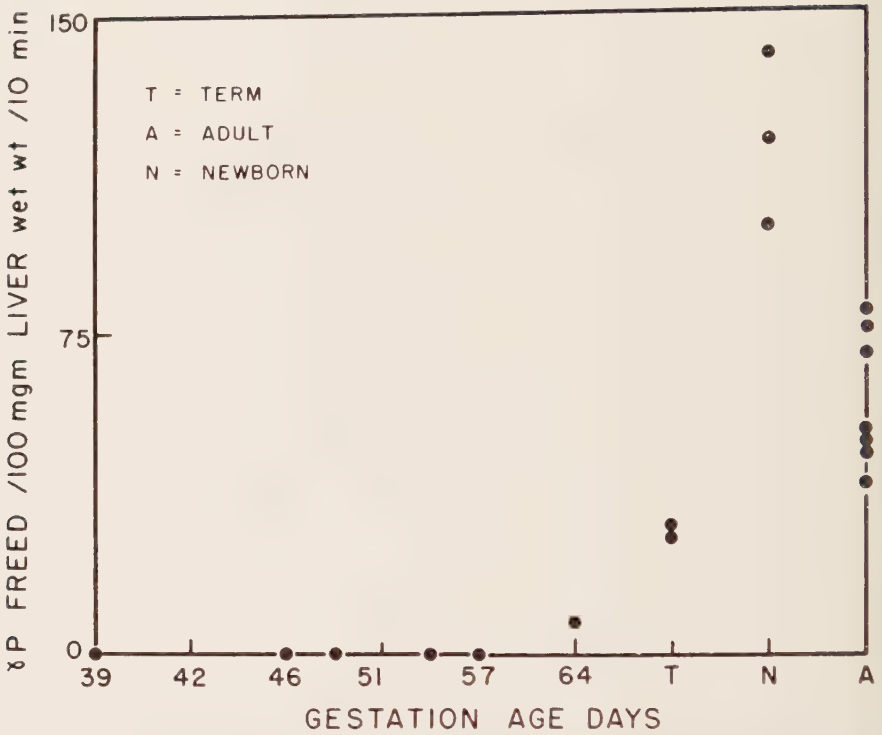

FIGURE 15. Glucose-6-phosphatase activity in fetal and adult liver. The activity of the enzyme is expressed in terms of amount of inorganic phosphate freed per unit time and per unit weight of liver from glucose-6-phosphate at pH 6.8. Newborn = three days postpartum. (From the original of figure 1. A. M. Nemeth. 1954. J. Biol. Chem. **208:** 773.)

a working hypothesis. In the case of the cerebral cortex, it will now be evident that cytological, biochemical, and functional differentiation are all closely related temporally and may represent the complex end results of the interaction of an inductive stimulus with the neuroblast. Cessation of growth of the nucleus at the critical period may be taken as evidence that a considerable degree of nuclear maturity precedes the beginning of rapid differentiation of the cytoplasm, signaled by the abrupt appearance and rapid elaboration of Nissl bodies and the intense sprouting of processes. This thesis, that the nucleus matures before clear-cut differentiation of the cytoplasm, is further substantiated by the appearance of a morphologically mature nucleolus during the critical period. The neuroplasm synthesized during prenatal development contains a constant amount of protein nitrogen[22] but, beginning at the critical period, an increasing amount of protein is directed to the synthesis of certain enzymes but not all of them. Some of the respiratory and glycolytic enzymes are among those the activity of which progressively increases after the critical period and, in accordance with this pattern, the rate of utilization and the rate of oxidation of glucose increase with maturation, thus indicating that increase in efficiency of production of useful energy is an essential part of the maturation of the nerve

cell. Functional differentiation, closely related in time to cytological and chemical differentiation, is evidenced by changes in fetal behavior and by the appearance of cortically induced muscular response and of a positive electroencephalogram. It appears that the electroencephalogram *may* be dependent upon alterations in the permeability of the cell membrane, permitting sodium ions to pass it.

So far as can be judged from the observations at hand, the pattern of enzyme changes which accompany the development of cerebral cortex and liver show marked differences and only slight similarities.

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Discussion of the Paper

Doctor Alexander G. Karczmar (*Sterling-Winthrop Research Institute, Rensselaer, N. Y.*): Some of the respiratory and glycolytic enzymes whose activity, as you have discovered, increases in the course of the cortical development are concerned with utilization of glucose. As, I believe, Doctor Flexner correctly surmised, the increase in the efficiency of the production of useful energy depends on the activity increment of this group of enzymes. He described also the increase of activity of brain cholinesterase at a moment roughly coinciding with the appearance of function. I should assume that he does not associate in his mind the increase of the activity of the respiratory and glycolytic enzymes with the appearance of function, and I should like to mention that it appears,

on the basis of a recent publication,* that the "efficiency" enzymes (in this case, succinoxidase) appear at the time of the increase of the efficiency of the energetics of the heart beat, but some time after the first heart beat.† I should like to ask him, however, whether he prefers to associate the appearance of function with that of cholinesterase, or does he think that a better correlation can be obtained between function and the alterations in Na permeability which he has also described in his paper?

Doctor Flexner: I would suppose that the appearance of function is likely based upon a complex series of events. Its correlation with a change in distribution of sodium, tentatively ascribed to a change in permeability to sodium, is more evident than its correlation with a change in activity of cholinesterase.

* Sippel, J. 1954. Exp. Zool. **126:** 205–233.
† *Op. cit.*, figures 1 and 2.

SUBSTRATE UTILIZATION IN CELL DIFFERENTIATION*

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During embryonic development, the cells of an embryo undergo progressive specialization. This specialization is evident in the morphological appearance and in the functional activity of the cell and, more subtly, in the developmental potentialities of the cell. This report is concerned primarily with the changes occurring in the functional activity of cells during the course of their development, particularly with those changes which determine the ability of the cell to synthesize simple substances. The changes that occur in synthetic ability during development are probably related in a causal way to subsequent developmental capacity—that is, to the prospective potency of a cell. The fact that the prospective potency of a cell is progressively restricted during development may well reflect a reduction or loss in the ability of the cell to carry out various types of syntheses. It seems apparent, however, that in the adult organism, some groups of cells carry on metabolic activities and synthesize various substances which are not detectable in the early embryo. Thus, during development, some cells may lose part of their original endowment, and others may acquire synthetic abilities not manifest in the early embryo. This generalization must be regarded with reservation, however, since present techniques do not permit a critical distinction between a latent and a nonexistent potentiality for a given synthesis.

Although the production of various complex molecules, such as the pituitary hormones of the adult, does not appear to be a detectable function of embryonic cells, it is not yet possible to prove that the production of such hormones represents genuinely new synthetic abilities—that is, those not present, even in a latent sense, in the type of cellular organization found in antecedent cells.

In support of the concept of differentiation by loss of synthetic ability is the observation that many enzymes are normally found in assayable quantities during the earliest stages of development (Moog, 1952), but that they tend to be concentrated preferentially in certain tissues or organs as development proceeds. That is, some enzymes are greatly reduced or lost in some tissues during functional differentiation. The observations of Ebert *et al.* (1955) on the progressive restriction of the areas of the chick blastoderm which synthesize the heart-muscle protein myosin likewise support the concept that biochemical differentiation involves losses of synthetic ability.

However, although antecedent embryonic cells may have the ability to carry out any of the syntheses performed by their adult daughter cells, it is apparent that all of the diverse daughter cells are not characterized by the same metabolic patterns, nor do they synthesize all of the substances manufactured by their sister cells. The cells of the thyroid, parathyroid, and thymus glands, as well as the various types of secretory and nonsecretory epithelial cells found

* This investigation was supported by the Michigan Memorial Phoenix Project No. 56 and by the Institute of Human Biology, University of Michigan, Ann Arbor, Mich. The assistance of Rubena Rabezzana and the collaboration of Doctor Glenn Fischer in part of this investigation is gratefully acknowledged.

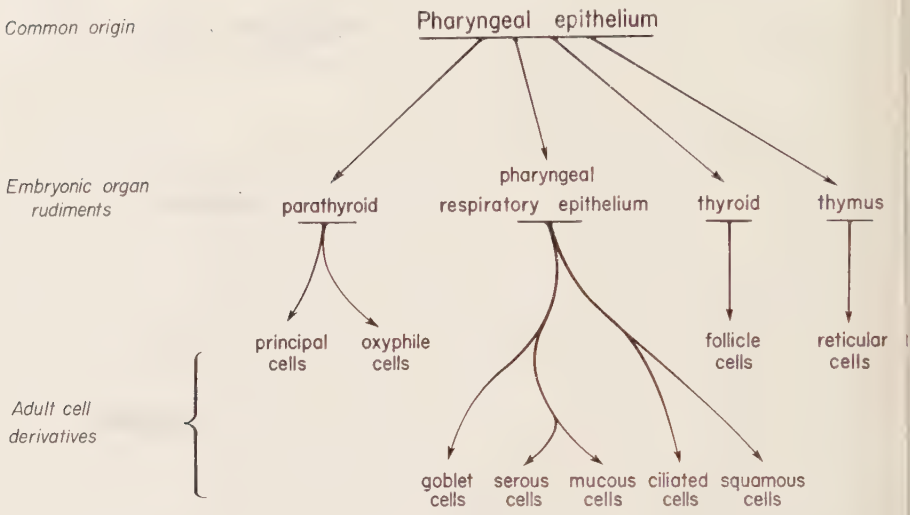

FIGURE 1. Diagram showing the developmental relationships of some of the derivatives of pharyngeal endoderm. The adult cell types possess distinctive synthetic abilities, and may differ also in their ability to synthesize simple metabolites.

in the lining of the pharynx and respiratory system, though all having a common endodermal origin, quite obviously have specialized synthetic abilities which distinguish them from one another (*cf.* FIGURE 1).

One plausible view of cellular differentiation pictures the cells of the organism as specializing in the synthesis not only of complex substances such as hormones or enzymes, but also in the synthesis of simple substances needed by them all. Thus a division of labor would be effected. Such simple substances as amino acids, purines, pyrimidines, *etc.*, may perhaps be synthesized by some cells and not by others. Since these substances are required by all cells, it is probable that all the cells at some early stage of embryonic development are equivalent in the capacity to synthesize them. As embryonic development progresses, the ability to synthesize these substances may become restricted to only certain types of cells, while the remaining cells are diverted to other synthetic jobs. Such losses of synthetic ability might well account for the apparently irreversible nature of differentiation. In any event, this hypothetical specialization in synthesis would seem to be an efficient way in which to organize the cellular activities of a metazoan. If such losses in synthetic ability do occur, and if they can be chemically defined, then the course of normal cellular differentiation might be controlled, since the chemical environment of the cells undergoing specialization could then be altered in a meaningful way. Efforts to test this hypothesis of the nature of cellular specialization have been concerned, in the investigations to be reported here, with: (1) the ability of differentiated or semidifferentiated cells of various types to synthesize simple substances such as amino acids, purines, or pyrimidines; and (2) the ability of melanoblasts of various genotypes to utilize particular substrates in the synthesis of their individual melanin pigments.

Metabolism of Glucose by Cells in Tissue Culture

In the first of these investigations, cells from organs of seven-day-old chick embryos were isolated and grown in tissue culture. Cells were taken from the liver, heart, skin, spleen, kidney, and the pigmented layer of the retina. These cells were grown in flasks or Petri dishes in the presence of a complex, chemically undefined medium consisting of chick embryo extract, chicken plasma, horse serum, Earle's saline (Earle, 1943), and amino acids or glucose labeled with C^{14}. After varying periods of growth in the presence of this medium, the cells were removed and fractionated, and the protein fraction was hydrolyzed with HCl. Aliquots of the hydrolysate were then chromatographed on filter paper in parallel runs. The distribution of radioactivity on the chromatograms was determined by counting with a Nuclear Scalar equipped with a G. E. open window end counter, and the development of the parallel chromatogram colorimetrically was used as the basis for a tentative identification of the sources of the radioactivity. When glucose was used as the labeled substrate, it was evident that liver and heart cells produced different pattern distributions of radioactivity on the chromatograms and that, therefore, they must have different patterns of synthetic ability. These differences are probably related to the incorporation of part of the glucose carbon skeleton into amino acids, particularly into alanine and aspartic acid, although other amino acids were also involved to a lesser extent.

Differences were also evident in the synthetic patterns of some of the other tissues but, as yet, no positive identification of the chemical differences has been made. These results are preliminary, and any conclusions drawn must be considered tentative pending the outcome of further and more detailed investigations that are now in progress. It may be noted, however, that results are available from other investigations that bear on this general problem. A. Fischer *et al.* (1953) have shown that chick heart fibroblasts grown in the presence of glucose labeled with C^{14} synthesize amino acids from metabolites ultimately derived from the glucose. The largest amount of radioactivity was found in alanine and aspartic acid, signifying a formation of pyruvate and oxaloacetate from glucose, with subsequent transamination to form the two amino acids. Some C^{14} was also found in the tissue serine, glycine, glutamic acid, and proline. In studies of protein synthesis and amino acid turnover in tissue culture, Gerarde *et al.* (1952) demonstrated that, in tissues maintained in Tyrode's medium containing glycine-1-C^{14}, the protein subsequently isolated was found to contain both labeled glycine and serine in the ratios of 3.6 for heart fibroblasts and 2.0 for lung cells. In similar experiments with DL-alanine 1-C^{14} and DL-phenylalanine-3-C^{14}, all of the C^{14} of the proteins was found restricted to those two amino acids (Winnick, 1952). Thus heart fibroblasts and lung cells differ in their ability to convert glycine to serine, but are alike in being unable to convert alanine or phenylalanine to any other amino acid.

Unfortunately, in none of these investigations have clones of cells been used. All have started with populations of cells derived, to be sure, from restricted areas of the embryo, but undoubtedly including cells of diverse synthetic

abilities. Negative results would have had little significance, since populations of cells drawn from different tissues might have the same pattern of synthetic abilities, although composed of a variety of cells of different, perhaps complementary, synthetic abilities. Since, in certain instances, however, these diverse populations of cells were shown to possess different synthetic capacities, certainly clones of cells extracted from the population would have shown similar or greater differences in synthetic ability.

The great difficulty in obtaining clones of cells from single cell isolations, in some instances, may be due to an obligate cross-feeding among closely associated cells. The provision of an adequate medium for such separated cells would be very difficult if the nutritive exchange consisted of labile substances. Much effort has been invested in several laboratories in the attempt to devise a chemically-defined medium which will sustain the indefinite growth of animal cells in tissue culture (*cf.* Waymouth, 1954, for review of the nutrition of animal cells). These efforts, though not yet successful, have made significant progress and, when such media are devised, they will greatly facilitate investigations of reciprocal nutritive relationships among the specialized cells of metazoans.

It is already evident, however, that a medium that may be satisfactory for one type of cell may be inadequate for another. A. Fischer (1953) for example, found that cardiac myoblasts were unable to survive on a diet that was entirely satisfactory to liver fibroblasts. He further observed that, in a medium containing a mixed culture of connective tissue cells and macrophages, the connective tissue cells gradually died out, leaving a pure culture of macrophages. The synthetic abilities of the fibroblasts may be more restricted therefore than those of the macrophages.

In studies on morphogenesis and differentiation in explanted chick blastoderms, Spratt (1950a, b) was able to demonstrate quantitative and qualitative differences in the nutritive requirements of different tissues and organs for carbohydrate substrates. Such differences in nutritional requirements are probably reflections of corresponding differences in synthetic abilities. Moreover, the nutritive requirements of the tissues changed, as differentiation proceeded, attesting to the changing patterns of synthetic ability during the course of differentiation.

All of these studies on the nutritive requirements of animal cells involved the use of complex, chemically undefined media. Such investigations would be facilitated and the interpretations made more certain if the media used were chemically defined. The availability of labeled compounds, however, has now, to some degree, circumvented the need for a chemically defined medium in testing the nutritive requirements and, as a corollary, the synthetic ability of cultured cells. With labeled compounds, the pathways of synthesis, interconversion, and degradation of amino acids, purines, pyrimidines, *etc.* may be followed, even though the compound in nonlabeled form is present in the medium, along with numerous unidentified constituents.

Underlying the approach discussed here is one assumption that must be regarded with reservation, namely, that explanted cells retain the pattern of metabolic syntheses which characterized them at the time of explantation.

There is evidence that the pattern of amino acid composition of cells remains constant *in vitro* (Gerarde *et al.* 1952), but the ability to synthesize the constituents of this pattern may not, of course, have remained constant. Nevertheless, this constancy of amino acid composition in cultured cells over an extended period of time is at least reasssuring, though it must be realized that significant changes in synthetic ability may occur immediately on explantation as a consequence of the isolation of the cells from the intact organism. It is also true that the synthetic activities of cells may vary with the type of medium in which they are grown. Fell and Mellanby (1953) have clearly shown that large quantities of vitamin A, added to the culture medium of various epithelial cell types, will induce the cells to assume the functions of a secretory epithelium and will inhibit keratinization.

Quite obviously, only the first steps have been taken in the investigation of the pathways of biochemical differentiation. Once the differences in synthetic ability now evident between related partly-differentiated cell types are clearly specified, then that portion of the study which is particularly significant for embryology may be undertaken: namely, the comparison of the patterns of syntheses found in the early embryonic cell types with those found in their specialized offspring.

Substrates for Melanogenesis

The second part of this general investigation deals with the rather special problem of melanogenesis. This investigation has already been carried far enough to answer specific questions and to raise several others. The problem simply is that of determining the substrates used in melanin synthesis by melanoblasts of different genotype. Melanin is found in all shades from yellow through brown to black. The particular color of melanin pigment synthesized by a melanocyte is governed by the genotype of the cell and the embryological history of the cell. The question which may be posed is: Do the different colors of melanin involve the use of different initial substrates in synthesizing the pigment, or are the colors attributable to different types of polymers of the same monomer or to associated elements or compounds which determine the specific color produced?

This problem was investigated by growing melanoblasts from mice or chicks in the presence of various possible labeled precursors of melanin. Both *in vivo* and *in vitro* techniques were used. Since it has been commonly believed that the amino acid tyrosine is the initial substrate for melanin synthesis, this compound, labeled in the second carbon atom of the side chain, was added to tissue cultures of embryonic chick skin. The skin contained melanoblasts in the process of making melanin. After melanocytes filled with melanin granules had differentiated, the tissue was fixed, sectioned, mounted on glass slides, and stained. Autoradiographs of the tissue were then prepared according to the following procedure. After staining with hematoxylin in water, the tissue on the slide was dipped in 4 per cent polyvinyl alcohol for a few minutes, and then the slide was allowed to dry for 12 hours while lying flat. Thus, the polyvinyl alcohol embedded the tissue and produced an optically smooth surface about

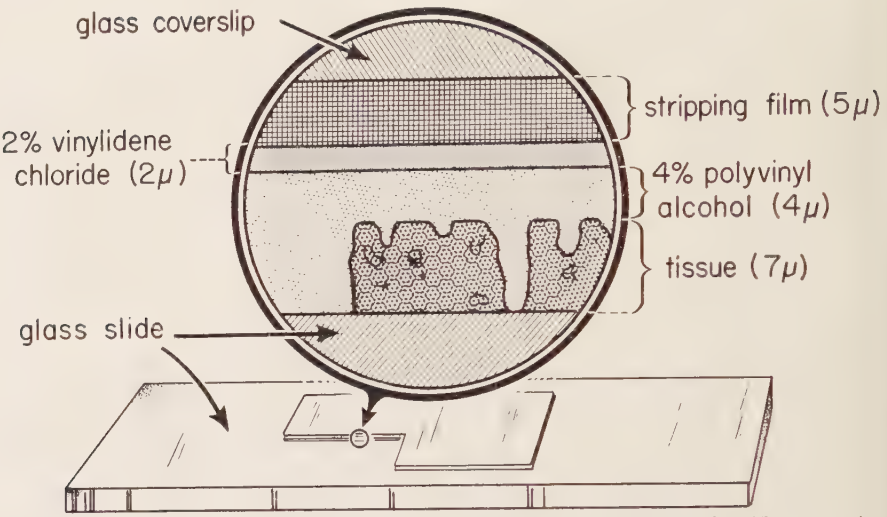

FIGURE 2. Arrangement and thickness of materials used in preparing autoradiographs. The preparation may be made permanent by mounting a coverslip on top of the film after it has been developed.

four μ above the tissue. Next, the slide was dipped in 2 per cent vinylidene chloride (saran), which deposited a chemically impervious layer about two μ thick. Finally, Kodak autoradiographic permeable base stripping film (about five μ thick) was floated onto the slide and allowed to dry in position above the tissue. Depending upon the level of radioactivity of the preparation, the film was exposed from one day to several weeks before being developed. After the film was developed, a coverslip was mounted on top of the film, and the tissue and its autoradiograph could then be viewed as a unit (FIGURE 2).

Autoradiographs prepared of tissues grown in media saturated with labeled tyrosine revealed that the tissue was radioactive, but the tyrosine was not selectively concentrated in the melanin granules (FIGURE 3). It is apparent that the radioactivity is distributed through the sectioned tissue in proportion to the concentration of the protoplasm, and no more radioactivity is lodged in the melanin granules than in the adjacent cytoplasm.

Another contender for the role of initial substrate in melanogenesis is 3-4-dihydroxyphenylalanine, or dopa the first stable product of the *in vitro* tyrosinase-catalyzed oxidation of tyrosine. Dopa is, in its turn, rapidly oxidized through various intermediates to melanin. Since dopa oxidase is more readily demonstrated in mammalian or avian tissue than is tyrosinase, many investigators have concluded that the initial substrate for melanogenesis, in these tissues, is indeed dopa rather than tyrosine. If dopa is the initial substrate, then of course, it must be formed by some mechanism other than the catalytic oxidation of tyrosine by tyrosinase.

Since labeled dopa was unavailable commercially at the time these experiments were begun, an attempt was made to produce labeled dopa from the labeled tyrosine through catalytic oxidation by tyrosinase in the presence of ascorbic acid. The presence of the ascorbic acid permits the accumulation of

FIGURE 3. Autoradiograph of skin containing melanocytes that develop *in vitro* in the presence of tyrosine labeled in the side chain with C^{14}. The photograph (right) of the autoradiograph is taken in the plane of the film about 10 μ above the tissue. The tissue is therefore out of focus, but the melanocytes can be located by reference to the photograph (left) taken with the tissue in focus.

FIGURE 4. Autoradiograph of melanocytes from a tissue culture of embryonic chick skin grown in the presence of radioactive oxidation products of tyrosine. The autoradiograph (right) was photographed by reflected light, thus making the silver grains appear as white specks. That the most intense localization of the radioactivity is in the melanocytes may be determined by comparing the photograph (left) of the melanocytes with the autoradiograph (right).

FIGURE 5 Autoradiograph of tissue containing a melanocyte that differentiated in the presence of dopa labeled with C^{14} in the side chain. There is no selective accumulation of radioactivity in the melanin granules. The outline of the melanocyte has been drawn on the autoradiograph (right) to facilitate comparison with the tissue photograph (left).

dopa in the reaction mixture (Krueger, 1950). The reaction mixture containing 2 ml. ascorbate at .67 mg. ml., 0.5 ml. DL tyrosine at 1 mg ml , and 0.1 ml. of a weak solution of a commercial tyrosinase was allowed to react until a distinct pink color became evident, indicating that some of the tyrosine had been converted to dopachrome. The reaction was then stopped by boiling. Chromatographic analysis revealed the presence of a small amount of dopa in the mixture. This mixture, containing a variety of melanin intermediates including dopa, was then added to cultures of chick melanoblasts, and autoradiographs taken of the culture after extensive melanization occurred. These autoradiographs revealed a distinct selective accumulation of radioactivity in the melanin granules, and these positive results were attributed initially to the small amount of dopa in the medium (FIGURE 4). After commercially produced dopa, labeled in the side chain, became available, these experiments were repeated with large concentrations of the dopa. Surprisingly, the autoradiographs prepared were completely negative so far as any selective incorporation of the dopa into melanin granules was concerned (FIGURE 5). Apparently, in the mixture of oxidation products of tyrosine, some melanin intermediate other than dopa was responsible for the positive autoradiographs obtained.

Since the tissue culture medium represents an artificial environment for the development of melanoblasts, it was desirable to check the results of the tissue culture experiments by corresponding experiments carried out on living animals. Consequently, labeled dopa and labeled tyrosine were injected subcutaneously into new-born C57 black mice and also yellow mice. Injections were made three times a day for four days, at which time the skin and hair of the mice appeared deeply pigmented. A total of about two microcuries was injected into each mouse. The skin was then removed, fixed, sectioned, and stained, and autoradiographs of the sections were prepared. Again, no selec-

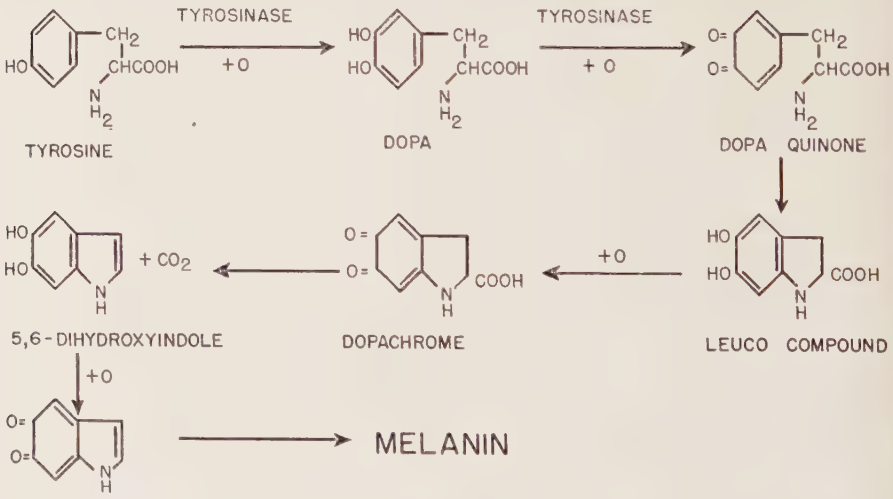

FIGURE 6. Commonly accepted scheme for the enzymatic oxidation of tyrosine to melanin. Note that the side chain of tyrosine is incorporated into the monomer that polymerizes to melanin.

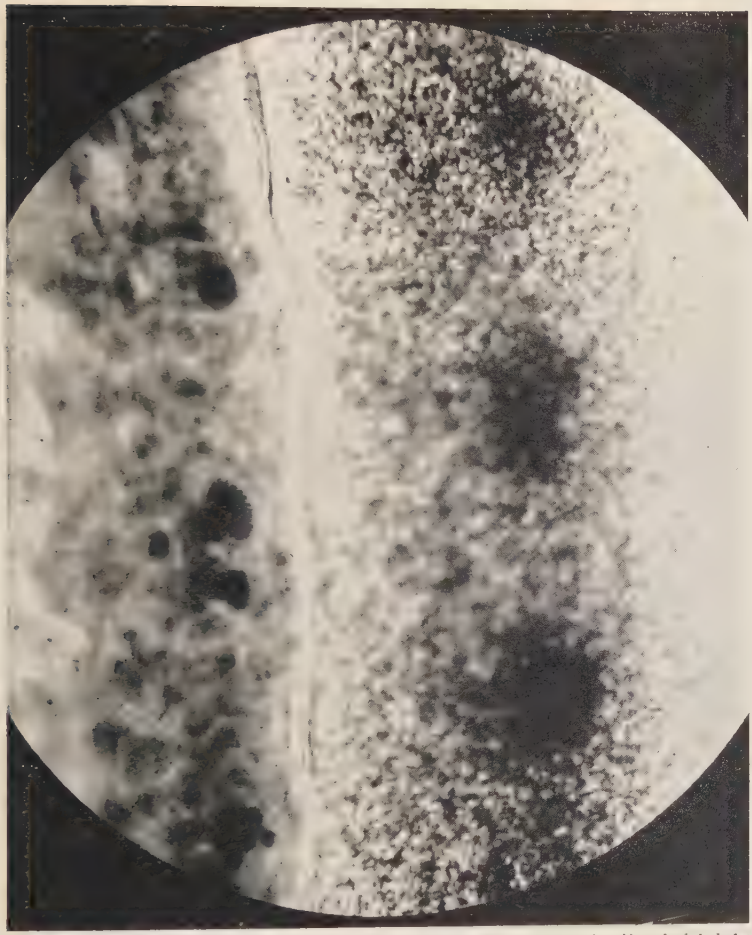

FIGURE 7. Autoradiograph of tissue and melanocytes grown in the presence of uniformly labeled tyrosine. The autoradiograph has been displaced obliquely to the right of the tissue. The melanocytes have rounded up, but are obviously the sources of intense radioactivity.

tive accumulation of the tyrosine or the dopa was evident in the melanin granules, even under these conditions of *in vivo* synthesis of the melanin. These results are difficult to reconcile with the commonly accepted scheme for melanogenesis starting with tyrosine or dopa (FIGURE 6).

The picture was further complicated by the results of experiments using uniformly labeled tyrosine, rather than tyrosine labeled only in the side chain. When the uniformly labeled tyrosine was added to tissue cultures of developing chick melanoblasts, the labeled material was selectively concentrated in the melanin granules, as shown by autoradiographs (FIGURE 7). This result implies that the side chain of tyrosine is not incorporated in the melanin, whereas the ring portion of the molecule does become a part of the melanin (*cf.* FIGURE 6). There is the difficulty, however, of explaining the previous results, in which some oxidation product of tyrosine labeled only in the side chain was

incorporated selectively into melanin granules. At present, no satisfactory explanation is at hand, and further experiments will be needed to clarify these seemingly contradictory results.

Since tryptophane had previously been implicated in the synthesis of a yellow pigment by mouse skin extracts (Foster, 1951), this amino acid labeled in the side chain was added to cultures containing developing chick melanoblasts. Not only did the tryptophane fail to yield selective autoradiographs of any part of the tissue but, in addition, it completely inhibited the synthesis of melanin without adversely affecting the general viability of the tissue cultures. Tryptophane would thus appear to be excluded as a substrate for melanogenesis in chick-tissue cultures.

Summary and Conclusions

(1) Semidifferentiated cells derived from different organs of the embryonic chick, when grown in tissue culture, exhibit different patterns in the metabolic transformation of glucose.

(2) Of several possible substrates which might be used by melanoblasts in the synthesis of melanin pigment, only uniformly labeled tyrosine and oxidation products of tyrosine were found to be differentially incorporated into melanin granules as shown by autoradiographs. The fact that the labeled side chain of neither tyrosine nor dopa served as melanin precursors in these tissue cultures, casts doubt upon the generally accepted scheme for melanogenesis based upon the action of tyrosinase upon tyrosine or dopa.

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THE ROLE OF PHENYLALANINE IN THE DIFFERENTIATION OF NEURAL CREST CELLS*

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Introduction

In the present experiments, the cells of salamander embryos from the middle gastrula to early tail-bud stages have been used as a starting point for studies of cellular differentiation. They are here considered, for the purpose of these experiments, to be undifferentiated. Such an initial point or state is a requisite for studies on cellular differentiation, since change in cellular function and structure through time is the essence of the differentiation process. The formal analysis of these morphological and physiological changes requires a precursor state from which the later cellular products are demonstrably different. It is possible to select any of several cellular states as precursors. For the present study, cells have been chosen from embryonic stages when all are essentially similar in terms of form and movement, as observed by tissue-culture methods. These cells show differences only as they undergo various differentiation processes through time. We shall be concerned, therefore, with changes in explanted cells from a state where all are apparently alike, both morphologically and in terms of their movements and migrations, to a state where visible differences in cellular behavior and form are apparent to the observer.

More particularly, we are concerned with cytoplasmic differences, since here characteristics which distinguish cell types most commonly appear. The cytoplasmic aspects of work concerned with cell differentiation are particularly germane in view of the recent experiments of King and Briggs (1954), who have reported that selected nuclei from cells beginning to undergo differentiation are capable of cooperating with the cytoplasm of amphibian eggs to bring about the development of a whole embryo. Without implying that a nuclear differentiation does not occur, it is possible, in the face of this evidence, to postulate a more determinative role of cytoplasmic components.

Finally, the work reported below concerns the differentiation of the urodele neuroepithelium, in particular its lateral edges, the neural crest. This tissue normally gives rise to pigment cells, ectomesenchyme, and subsequent head skeletal products, sensory ganglion cells, and the adrenal medulla (see Horstadius, 1950, for a modern review). In histiotypic cultures in nutrient medium, the differentiation of melanophores, xanthophores, ectomesenchyme (Schwann cells, fibroblasts, and melanophages) and colorless stellate "ganglion" cells can be closely observed from this tissue. Pigment cells develop a granular melanoprotein. The melanin moiety has been presumed to be derived from a metabolic sequence utilizing tyrosine or phenylalanine. Epinephrin, a hormone of adrenal medullary cells, appears to have the same precursor metabolites. Willmer (1951) pointed out that both cell types were derived from neural crest and then raised the question as to whether all cells of neural crest origin might have a similar metabolism. We have accepted the discussion of

* This investigation has been aided by a grant from the National Science Foundation, Washington, D. C.

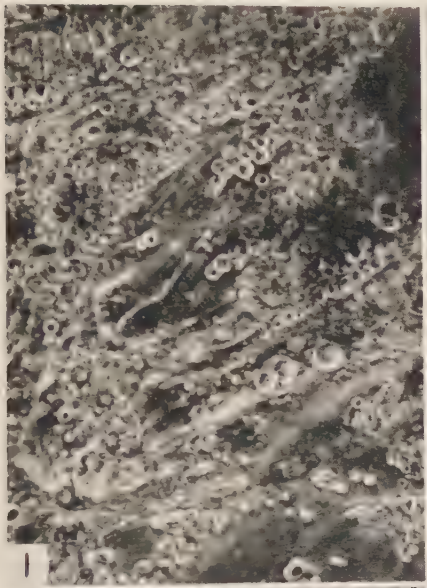
Willmer as a start for a working hypothesis as to the mechanism of neural crest cell differentiation. To do this, we have added a causal postulate, namely that a special metabolism of phenylalanine or tyrosine is necessary for the normal differentiation of neural crest cells. Should this metabolic sequence be disturbed sufficiently, differentiation of neural crest cells, specifically pigment cells and ectomesenchyme, would not occur.

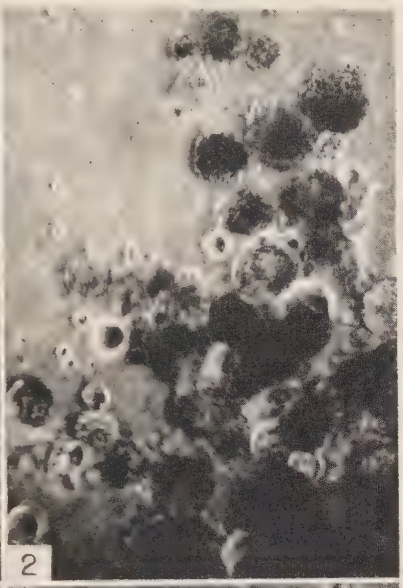
Materials and Methods

Techniques for the precise explantation of pure neuroepithelium, free from all other cells have been developed (Wilde, in press). Over 340 such explants from *Ambystoma maculatum* (Shaw), stages $11\frac{1}{2}$ to 21, were made to a nutrient medium which supports the normal differentiation of these cells (Wilde, 1948, 1950, 1952, 1951, 1953, and in press). The explants were mounted on glass to foster histiotypic development of the explant. To the medium were added certain structural analogues of phenylalanine and tyrosine at a maximum concentration of 1.0 millimoles/L. The following structural analogues were used: o-fluorophenylalanine 1.0, 0.5, 0.25, 0.125 millimoles/L.; m-fluorophenylalanine 1.0 millimoles/L.; p-fluorophenylalanine 1.0 millimoles/L.; B-3 thianaphthylalanine 1.0 millimoles/L.; 3-aminotyrosine 0.5 millimoles/L.; B-2 thienylalanine, 1.0 millimoles/L.; B-3 thienylalanine 1.0 millimoles/L.; B-2 furylalanine 1.0 millimoles/L.; phenyl lactic acid 1.0 millimoles/L. All compounds were of the DL type. The basal medium was supplemented with 1.0 millimoles/L. of either phenylalanine or tyrosine after experiments showed that no difference in rate or degree of differentiation of neuroepithelium could be observed in cultures in basal medium compared with those in medium supplemented with the normal amino acids.

The effects on the differentiation of the neural crest cells which would be required to establish the validity of the working hypothesis were that neural crest cells fail to differentiate in some measure in the presence of the analogues. The demonstration of differentiation failure of this degree of specificity further required that cells of other fates in differentiation be unaffected by the inhibitory analogues. The control designed to test specificity of effect against neural crest differentiation was to include, with certain explants of neuroepithelium, a small portion of somite mesoderm. Under control culture conditions, added somite mesoderm differentiated into striated muscle of great morphological perfection (FIGURE 1). An inhibitory effect of phenylalanine or tyrosine analogues was considered to be specific against neural crest differentiation only when somite mesoderm in the same culture differentiated into myoblasts and striated muscle.

Experiments attempting to elicit a neural crest type of cellular differentiation were carried out by explanting pure ventral ectoderm or ventral ectoderm with a small number of ventral internal yolky cells (stages $11\frac{1}{2}$ to 19) to culture medium supplemented with 1.0, 2.0, 4.0, and 8.0 millimoles/L. phenylalanine. Controls for this experimental series were placed in basal medium without supplementation. Under control conditions, explants of pure ventral ectoderm grow as sheets of epithelial cells. With few internal yolky cells present, leucocytes and some mesenchyme appear.





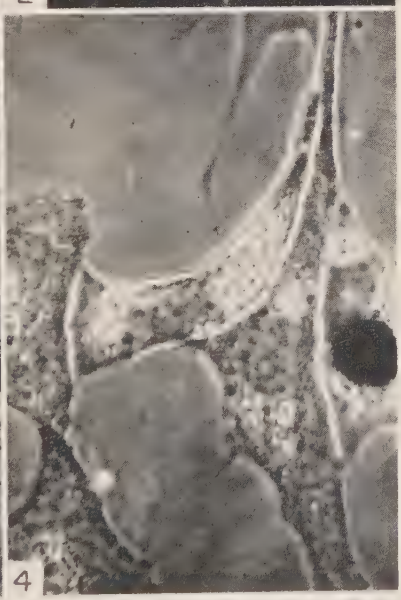


FIGURE 1. Mature striated muscle in a tissue culture of neuroepithelium and somite mesoderm. Explanted at stage 16 and cultivated for 19 days in nutrient medium supplemented with 1.0 millimoles L. phenylalanine. Phase contrast, ×300.

FIGURE 2. Undifferentiated ameboid cells in a tissue culture of neuroepithelium. Explanted at stage 13 and cultivated for 5 days in nutrient medium supplemented with 1.0 millimoles/L. o-fluorophenylalanine. The cells did not differentiate. Phase contrast, ×300.

FIGURE 3. Normal differentiation of pigment cells and ectomesenchyme in a tissue culture of neuroepithelium. Explanted at stage 18 and cultivated for 13 days in basal medium supplemented with 1.0 millimoles L. phenylalanine. Compare with FIGURE 2. Phase contrast, ×300.

FIGURE 4. Normal early differentiation of pigment cells in a tissue culture of neuroepithelium. Explanted at stage 15 and cultivated for 7 days in nutrient medium supplemented with 5.5 millimoles L. o-fluorophenylalanine and 0.5 millimoles L. phenylalanine. The inhibition of differentiation exemplified in FIGURE 2 has been relieved. Phase contrast, ×300.

Twenty-four hours following explantation to all media used, the explants lie against the glass of the cover slip and a small number of moving free cells surrounds them. The cells are all similar and ameboid. They move by means of broad lobopodia. This type of activity is characteristic of all cells that fall in the present category of undifferentiated cells. Cells that retain this state for 72 hours or more either fail to undergo any differentiation or show a retardation of rate of differentiation and associated abnormal phenomena. The onset of differentiation is characterized by a change in the form and migratory habits of the cells, with the production of multiple slender filopodia. FIGURE 2 represents undifferentiated cells with their lobopodia. The normal differentiation of explanted neuroepithelium in control media is exemplified in FIGURE 3.

Results

Three types of inhibitory effects on neural crest cell differentiation were obtained in the experiments. These effects were directly correlated with the type of structural change by which the analogue molecule differed from phenylalanine or tyrosine. Where additions of atoms or groups had been made to the phenyl ring of phenylalanine, a general inhibition of all cells exposed to the compound occurred. The inhibition varied from a complete failure of differentiation to a general retardation of rate of differentiation. The series of compounds was effective in the following order: o-fluorophenylalanine > B-3 thianaphthylalanine > m-fluorophenylalanine = p-fluorophenylalanine, where the molarities were constant at 1.0 millimoles L. 3-Aminotyrosine was toxic at the level tested (0.5 millimoles/L.).

Where the phenyl group had been replaced by a heterocyclic, 5-membered ring, a specific inhibition of ectomesenchyme differentiation from the neural crest occurred. Pigment cell differentiation was unaffected. Striated muscle differentiation from added somite mesoderm was also unaffected.

Where the α amino group had been removed and replaced by an hydroxyl (phenyl lactic acid), pigment cell differentiation failed. Ectomesenchyme differentiation from neural crest was unaffected. Striated muscle developed in a normal manner from somite mesoderm when this material was included in an explant of neuroepithelium exposed to culture medium supplemented with 1.0 millimoles/L. of phenyl lactic acid.

General inhibitory phenomena. The most dramatic effects in inhibition of differentiation were brought about by the addition of 1.0 millimoles L. o-fluorophenylalanine to basal medium. Under these conditions, explants of neuroepithelium or neuroepithelium with somite mesoderm underwent no differentiation. Cells migrated from the explants in large numbers, but remained ameboid, progressing by means of large lobopodia. After five days *in vitro*, a gradual morbidity of the cells was seen until, at 10 to 12 days, few actively moving cells remained. Regardless of this phenomenon, no cell ever showed any recognizable degree of differentiation. FIGURE 2 is representative of cases where neuroepithelial cells fail to differentiate in nutrient medium supplemented with 1.0 millimoles L. o-fluorophenylalanine. With the exception

of diminution of yolk, the cells are morphologically identical with cells at the time of explantation.

Experiments to test the concentration of o-fluorophenylalanine, which was effective in bringing about complete inhibition of differentiation, showed that, at 0.5 millimoles L., no differentiation occurred. At 0.25 millimoles/L., there was a marked delay in rate of differentiation, but all types of differentiated neural crest derivative cells ultimately appeared. Striated muscle developed slowly from somite mesoderm cells when they were included in an explant under these conditions. A concentration of 0.125 millimoles L. had no effect upon the differentiation of neural crest or somitic cells These cultures were indistinguishable from controls in basal medium.

The inhibitory effect of o-fluorophenylalanine was not shown when explants of neuroepithelium or neuroepithelium and somite mesoderm were cultured in basal medium supplemented with 0.5 millimoles L. of o-fluorophenylalanine and 0.5 millimoles L. of phenylalanine. FIGURE 4 is a representation of such a culture. The removal of the severe inhibitory effects on differentiation of o-fluorophenylalanine by an equimolar amount of phenylalanine indicated that this inhibition of differentiation was specifically associated with a metabolic sequence in which phenylalanine is a substrate.

The m-fluoro- and p-fluoro- analogues of phenylalanine, when included in nutrient medium containing explants of neuroepithelium, with or without somitic cells (1.0 millimoles L.), acted as general inhibitors of differentiation of all cell types, but were much less severe in their effects than o-fluorophenylalanine. The inhibition was reflected in a slowing of the rate of differentiation comparable in all respects to that of o-fluorophenylalanine at a level of 0.25 millimoles L. in the nutrient medium. Under supplementation with either m-fluoro or p-fluorophenylalanine, pigment cells and ectomesenchyme cells differentiated slowly, but completely. Striated muscle differentiated slowly from somitic cells when they were included in the explant.

A fourth compound which must be included in the general inhibitory series is B-3 thianaphthylalanine. This compound differs from phenylalanine in that a thianaphthyl group replaces the phenyl group of the natural metabolite. When pure neuroepithelium or neuroepithelium with somite was cultured in nutrient medium supplemented with 1.0 millimoles L. B-3 thianaphthylalanine, severe inhibitory effects on the differentiation of the explanted cells became apparent. The cells migrated from the explant, but never completed their differentiation. All cells remained plump and rounded. While they developed so far as to lose, in part, their lobopodia, their filopodia were very short and stubby. A few of these cells became questionably dendritic with but two or three processes. These cells later developed a mild degree of pigmentation in no way comparable to the degree of pigmentation of melanophores in control cultures. The short filopodia were characteristic only of cells cultured in the presence of B-3 thianaphthylalanine. Myoblastlike cells also developed from somite when this material was included in cultures with neuroepithelium.

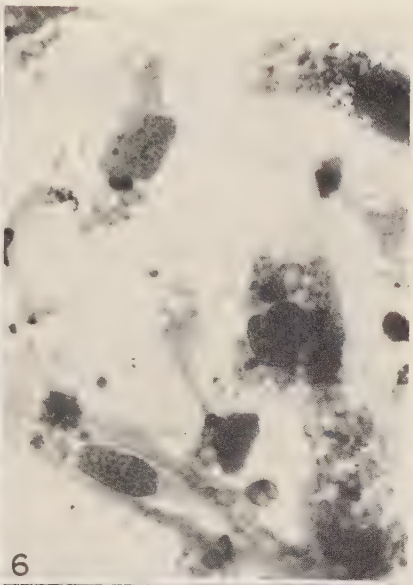
The last compound of the general inhibitory group, 3-aminotyrosine, proved to be the most toxic of all compounds tested. At a level of 0.5 millimoles L., 3-aminotyrosine brought about a rapid mortality of the neuroepithelial cells

explanted. After five days *in vitro*, few viable moving cells were present. These underwent no differentiation. Inclusion of both 0.5 millimoles/L. 3-aminotyrosine and 0.5 millimoles/L. phenylalanine did not relieve the toxic effect of the compound on the explanted cells. Apparently the toxicity of 3-aminotyrosine and its inhibition of the differentiation of the few viable cells is not removed by the presence of equimolar amounts of phenylalanine.

To summarize at this point, a general inhibitory effect on the differentiation of both neuroepithelium and somite cells is brought about when these cells are cultivated in nutrient medium supplemented with analogues of phenylalanine to whose phenyl rings additions have been made. In one instance (o-fluorophenylalanine, the most severe nontoxic inhibitor), the inhibition is removed by the presence in the medium of an equimolar amount of phenylalanine.

Specific inhibitory phenomena. Inhibitions found in cultures of neuroepithelium supplemented with analogues whose phenyl groups had been replaced by a 5-membered heterocyclic ring were strikingly different from the general inhibition of differentiation reported above. Here specific inhibitory phenomena were obtained. In cultures of neuroepithelium explanted in nutrient medium supplemented with 1.0 millimoles L. of B-2 thienyl, B-3 thienyl, or B-2 furylalanine, no ectomesenchyme cells differentiated. Differentiation of pigment cells was unaffected. The lack of ectomesenchyme cells gave a clear appearance to the cultures, with rather evenly spaced melanophores and xanthophores surrounding the explant. No Schwann cells were found along nerve axones as a consequence of the failure of ectomesenchyme differentiation (FIGURE 5). Examination of the explant itself indicated that the failure was not due to formation of a nonmigratory ectomesenchyme. There was no apparent increase in the number of pigment cells, nor was the number of nonpigmented stellate "ganglion cells" markedly increased. Fibroblasts, Schwann cells, and melanophages, the cells normally constituting ectomesenchyme in control cultures, were absent. When somite material was included with neuroepithelium in this series of experiments, there was no impedance of the differentiation of striated muscle. Striated muscle developed at a rate strictly comparable to its rate of appearance in cultures of the same type placed in control medium. The replacement of the phenyl group of phenylalanine by a 5-membered heterocyclic ring and the incorporation of such compounds in nutrient medium appeared to inhibit specifically the differentiation of ectomesenchyme from the explanted neuroepithelium. It would appear that the phenyl group of phenylalanine is particularly necessary in the metabolic sequences leading to differentiation of ectomesenchyme.

Phenyl lactic acid is the compound obtained by replacing the α amino group of phenylalanine with an hydroxyl. It is an occasional abnormal byproduct of phenylalanine metabolism in mammals. When neuroepithelium was explanted to nutrient medium supplemented with 1.0 millimoles/L. phenyl lactic acid, no normal pigment cells differentiated (FIGURE 6). While, in the later stages of these cultures, there was a very light irregular pigmentation of all the ectomesenchyme cells which did differentiate, neither melanogenesis nor morphogenesis of pigment cells occurred. The usual complement of colorless,



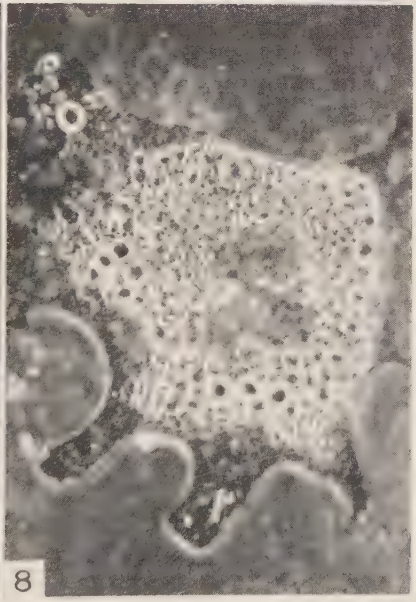


FIGURE 5. Failure of ectomesenchyme differentiation in the presence of normal pigment cell differentiation in a tissue culture of neuroepithelium. Explanted at stage 14 and cultivated for 13 days in nutrient medium supplemented with 1.0 millimoles L B 3 thienyl-alanine. Compare with FIGURE 6. Phase contrast, ×300.

FIGURE 6. Failure of pigment cell differentiation in the presence of normal ectomesenchyme and myoblast differentiation in a tissue culture of neuroepithelium and somite mesoderm. Explanted at stage 21 and cultivated for 10 days in nutrient medium supplemented with 1.0 millimoles/L. phenyl lactic acid. Compare with FIGURE 5. Hematoxylin and fast green, ×300.

FIGURE 7. Anomalous pigment cell elicited in an explant of ventral ectoderm with a few internal yolky cells. Explanted at stage 14 and cultivated in nutrient medium supplemented with 1.0 millimoles/L. phenyl alanine for 18 days. Phase contrast, ×300.

FIGURE 8. Normal (neural crest-like) pigment cell elicited in a tissue culture of pure ventral ectoderm. Explanted at stage 12 and cultivated for 7 days in nutrient medium supplemented with 8.0 millimoles/L. phenyl-alanine. Compare with FIGURES 3, 4, 5, and 7. Phase contrast, ×300.

stellate "ganglion cells" appeared, but no cells of the normal dendritic form characteristic of pigment cells differentiated. When somite cells were included in an explant with neuroepithelium in the presence of 1.0 millimoles/L. of phenyllactic acid, differentiation of striated muscle was undisturbed. It would appear that the metabolism concerned with differentiation of explanted pigment cells and their coordinate pigmentation requires, in particular, the presence of the α amino group of phenylalanine.

Elicitation of neural crestlike cell differentiation. At this point we may sum up the evidence with regard to the role of the aromatic amino acids, phenylalanine and tyrosine, in the normal differentiation of cells from the neuroepithelium. The addition of atoms or groups to the phenyl ring results in compounds which are general inhibitors of cell differentiation of all types. This inhibition is not limited to the neural crest. The inhibitory effect of one of these compounds, o-fluorophenylalanine, is released by an equimolar amount of phenylalanine. Replacement of the phenyl group by a 5-membered heterocyclic ring results in compounds which act as specific inhibitors of ectomesenchyme differentiation, while the replacement of the α amino group of phenylalanine by an hydroxyl results in a compound that specifically inhibits the differentiation of pigment cells. The latter two effects are specific for the neuroepithelium, since striated muscle differentiation from somite cells is unaffected.

It seems justifiable to conclude that phenylalanine is a necessary metabolite for the chemical processes that are involved in the differentiation of neural crest cells in tissue culture. If this situation is equally true for the intact embryo, then it must be postulated that a sufficient concentration of phenylalanine exists in the region of the developing neural crest at the time these differentiations normally take place. Conversely, a phenylalanine concentration proper for neural crest differentiation should not be present in other parts of the embryo where the type of differentiation peculiar to the neural crest does not take place. However, a neural crestlike differentiation might be demonstrated in nonneural crest cells if they were presented with a suitable concentration of phenylalanine at a suitable time. This reasoning has been tested by the following experiments.

Pieces of pure ventral ectoderm or ventral ectoderm with a few internal yolky cells of stages $11\frac{1}{2}$ to 19 were explanted to nutrient medium supplemented with 1.0, 2.0, 4.0, and 8.0 millimoles/L. phenylalanine. Control explants of identical source were cultivated in basal medium.

Under control conditions, ventral ectoderm developed in culture as an expanding sheet of epithelial cells. No pigment cells were formed, and there was no melanogenesis. Only when internal yolky cells were included with ventral ectoderm did free cells appear. These differentiated as leukocytes and mesenchyme cells.

When an explant of pure ventral ectoderm was cultivated in nutrient medium supplemented by 1.0 millimoles/L. phenylalanine, free migrating cells appeared. These cells were large and contained many large yolk platelets. As the culture matured, the cells lost their lobopodia and developed numerous fine filopodia. However, they did not become dendritic, although they could exhibit either

spindle or somewhat stellate shapes. Many of these cells became pigmented (FIGURE 7). The melanogenesis seen in these cells was at first perinuclear, where melanin granules of homogeneous size appeared. The cells continued to increase their pigment load and became very dark. Rarely, however, did the granular pigment extend into the fine processes of the cells, but it remained evenly dispersed within the rounded body of the cell. This anomalous pigmentation was characteristic of free cells of origin in ventral ectoderm when cultured in the presence of 1.0 millimoles L. phenylalanine. Some cells, retained on the surface of the explant, developed the same pigmentation.

When ventral ectoderm was cultivated in nutrient medium supplemented with 2.0, 4.0, or 8.0 millimoles L. phenylalanine, cells essentially indistinguishable from those normally differentiating from the neuroepithelium appeared (FIGURE 8). Along with these elicited pigment cells, many cells exhibited pigmentation of the anomalous type discussed above. The neural crestlike cells were large and dendritic. An initially perinuclear pigmentation spread evenly throughout their cytoplasm and extended into the processes of these cells. Only the relatively larger amount of yolk indicated their ventral origin. The elicitation of melanophores occurred equally well in cultures supplemented with 2.0, 4.0, or 8.0 millimoles/L. phenylalanine. The last concentration approached the toxic level for phenylalanine under these conditions.

Many large spindle cells with much yolk and fine filose processes appeared in the cultures. They did not become pigmented. It is difficult to equate these cells with an elicited ectomesenchyme in view of their large size and heavy yolk load, but they did adopt the habit of ectomesenchyme, both in migratory activity and in over-all morphology.

It can be concluded from these experiments that relatively low concentrations of phenylalanine, included as a component of a medium in which pure ventral ectoderm has been placed, can elicit the differentiation of cells similar to those normally derivable from the edges of the neuroepithelium.

Discussion

The experiments presented have demonstrated that the chemical events whereby neural crest pigment cells and ectomesenchyme cells become different from their antecedents are dependent upon the presence of a sufficient concentration of phenylalanine. Therefore, the causal postulate set up in the working hypothesis has been satisfied. Not only does phenylalanine appear to be essential for neural crest differentiation, but certain parts of the molecule are peculiarly essential for particular types of neural crest differentiations. Thus the α amino group of phenylalanine is essential for the differentiation of pigment cells and pigmentation. In the absence of the α amino group, the rest of the molecule is ineffectual in the metabolism through which pigment cells differentiate. Lerner *et al.* (1949) have demonstrated in this connection that tyrosinase activity in extracts of Harding-Passey mouse melanoma is inhibited by analogues of tyrosine where the α amino group has been altered by the addition of a formyl or acetyl group.

The phenyl ring of phenylalanine appears to be the essential portion necessary for the special metabolic sequences whereby ectomesenchyme differen-

tiates. The presence of similar molecules without the phenyl group is ineffectual in mediating ectomesenchyme differentiation. Finally, phenylalanine itself can elicit from nonneural crest cells a type of differentiation essentially similar to that normally found in the neural crest.

Here a relatively simple, low-molecular weight compound, which presumably acts as a substrate, has been shown to play a key role in the chemical processes concerned with specific cell differentiations. The only other instance where a known compound has been demonstrated to mediate a specific type of cell differentiation in animals is in the case of vitamin A (Fell and Mellanby, 1953; Weiss and James, 1952). Vitamin A in excess can convert keratinizing epithelium of the chick to ciliated, mucous secreting (rhinal) epithelium.

If substrate compounds or ratios of substrates do play a key role in the metabolism of cell differentiation, the proper substrate ratios must exist in time and place within the embryo in order for normal developmental sequences to occur. This concept would describe the embryo as a kinetic system in which different presumptive regions are characterized by differing concentrations of ratios of metabolites. The artificial or experimental disruption of normal local substrate ratios would bring about differentiations of unexpected types. The elicitation of neural crestlike cell differentiations from ventral ectoderm by phenylalanine can be tentatively interpreted in this light. Further experiments on the metabolic system of neural crest differentiation and other differentiation systems are necessary before the substrate localizations concept can be held valid.

Conclusion

Experiments to test the hypothesis that a special phenylalanine or tyrosine metabolism is essential for the normal differentiation of pigment cells and ectomesenchyme cells from the neuroepithelium have been carried out by means of tissue cultures where the medium has been supplemented with analogues of phenylalanine or tyrosine. The results indicate that phenylalanine is essential for the differentiation of both pigment cells and ectomesenchyme cells. The α amino group of phenylalanine is essential for the differentiation of pigment cells, while the phenyl ring is essential for ectomesenchyme differentiation.

Phenylalanine itself, in low concentration, can elicit from pure ventral ectoderm the differentiation of anomalous pigment cells, pigment cells indistinguishable from those of the neural crest and a strange mesenchyme.

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Part II. Immunobiological Approach to Problems of Differentiation

THE ROLE OF GENES AND CYTOPLASMIC PARTICLES IN DIFFERENTIATION*

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Introduction

The problem of differentiation has been frequently posed by asking how it comes about that the different cells of a multicellular organism become morphologically and physiologically distinct although presumably containing the same genes in their chromosomes. On the other hand, differentiated functional cells belonging to different species and even phyla of animals which may be assumed to be genetically very different may be very similar to each other if they perform the same function; *e. g.* nerve cells and muscle cells from widely different groups of animals may resemble each other profoundly. Nevertheless, good evidence exists that their cytological structure and chemical make-up is under genic control. This fact is clearly shown by the genetically controlled differences in the immunological properties of differentiated cells in the same species, of which the many inherited variations of red blood cells in man present a good example.

The functional structures of differentiated cells, such as muscle fibrils and secretory granules, are cytoplasmic structures. The general question arises, therefore, how genes determine the characteristic form and constitution of the cytoplasmic structures and how, on the other hand, cytosplamic structures of a similar kind can be formed under the influence of widely different genotypes.

Strain Differences of Mitochondria in Mice

In order to attack this problem more concretely, a particular type of cytoplasmic structure has been investigated, the mitochondria. These structures appear to be of particular interest since they are ubiquitous cytoplasmic organels found in almost all cells investigated. It has been shown by a large number of investigations (Schneider and Hogeboom, 1951; Schneider, 1953) that these structures contain a number of important enzymatic systems of the cell. There are significant morphological differences between the structures of the mitochondria in different types of cells, probably related to their function. The interaction between nuclear genes and mitochondrial structure and function seems therefore a problem of fundamental interest.

The first question to be investigated is whether differences in the mitochondrial makeup of homologous cells from different strains can be demonstrated. For this purpose, two highly inbred mouse strains, C57 Black and Bagg albino (BALB), were employed. The first method used for the demonstration of a difference between the liver mitochondria of these two strains was immuno-

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logical. Mitochondria from the livers of BALB males were isolated by centrifugation in .88 M sucrose at 24,000 × g., according to the method of Hogeboom, Schneider, and Pallade (1948). Rabbits were immunized against these preparations by three injections spread over four days, using *Staphylococcus* toxin as an adjuvant (Maculla, 1947). The sera were inactivated and the Forssman antibodies removed by absorption with red sheep cells. For the demonstration of antibodies, the quantitative complement fixation method was used, since it is known that isolated mitochondria agglutinate spontaneously in saline (Dianziani, 1951). Preparations of liver mitochondria from C57 and BALB males, adjusted to contain 0.04 or 0.05 mg. N per ml., were used as antigens. This concentration of antigen was employed because it has been shown that it does not exert any hemolytic or anticomplementary activity in either antigen. The antigens, to which different dilutions of antiserum and six 50 per cent units of guinea pig complement were added, were kept in the refrigerator overnight with or without previous incubation at 37° C. Sensitized red sheep cells were added and the tubes incubated for 30 minutes at 37° C. In order to determine the degree of hemolysis obtained, the reaction tubes were centrifuged, and the amount of hemoglobin released was measured in a Coleman Junior spectrophotometer at 550 mμ. The results are given as per cent total hemolysis.

Most sera reacted at a higher titer with the homologous antigen BALB than with the heterologous C57 antigen. This reaction indicates that there is some kind of a difference between the isolated mitochondria of the two strains. An exception is offered by our serum 28, which reacted consistently at a higher titer with the heterologous antigen C57 than with the homologous antigen, BALB. This result can best be interpreted by assuming that this serum contains particularly strong antibodies against an antigen which is common to BALB and C57 mitochondria, but present in larger amounts in preparations from C57. On the whole, then, these serological observations indicate that there exists a difference between BALB and C57 Bl liver mitochondria, and that this difference affects at least two components. One consists in a quantitative increase of a common antigen in C57, while the other may be either a qualitative difference, or a quantitative difference in favor of BALB.

These conclusions have been borne out by chemical investigations of the isolated liver mitochondria from the two strains. It has been shown that mitochondrial preparations obtained from males of these two mouse strains can be distinguished by their phosphorus nitrogen ratios (Caspari and Santway, 1954). Using mitochondrial preparations from 76 BALB males and 75 C57 males, it could be demonstrated that their average P/N ratios are significantly different, being 96.26 ± 0.96 mg. P/gN in BALB and 102.03 ± 0.65 mg. P/gN in C57. It was furthermore shown that at least part of this difference between the two strains is due to their differential content of relative amounts of ribose nucleic acid and protein.

It has been shown by Chantrenne (1947), Novikoff *et al.* (1953), Kuff and Schneider (1954), and Paigen (1954) that mammalian isolated mitochondria can be divided by differential centrifugation into a number of subfractions of different composition and enzymatic function. All authors agree that the

TABLE 1

CONTRIBUTION OF DIFFERENT MITOCHONDRIAL FRACTIONS FROM BALB AND C57 MALES TO THE TOTAL P/N RATIO, EXPRESSED IN MG. PHOSPHORUS/g. TOTAL NITROGEN. MEANS OF 19 PREPARATIONS FOR EACH STRAIN

	MT1	MT2	MT3	Total mitochondria (calculated)	Total mitochondria (observed)
BALB	24.48	33.53	38.10	96.11	95.14
C57	25.10	35.66	40.37	101.13	101.48
Difference	0.62	2.13	2.27	5.02	6.34

particles sedimented by low centrifugal forces have a lower P/N ratio than those that are obtained with higher centrifugal forces. A difference in P/N ratio between different strains, as it exists between BALB and C57, could therefore be due either to a qualitative difference in some or all of the fractions, or to a relatively larger amount of the mitochondrial fraction sedimented by high centrifugal forces in the strain C57. In order to distinguish between these possibilities, mitochondria from both strains were isolated in .88 M sucrose and divided into three fractions containing about equal amounts of material, by centrifugation at 3,000 × g., 6,000 × g. and 24,000 × g. Phosphorus and nitrogen were determined in all three fractions, as well as in the original isolated mitochondrial material. It turned out that in the two fractions sedimented at 3,000 × g. and 6,000 × g., C57 had a significantly higher P/N ratio than BALB; there was no evidence for any difference in the 24,000 × g. fraction. When, however, the contributions of the three fractions were compared on the basis of their nitrogen content, expressed as per cent of total nitrogen, a difference at about the 5 per cent level of significance was found in the 24,000 × g. fraction, and this difference was in favor of C57. The strain differences in the two heavier fractions were not significant. The values for phosphorus and nitrogen obtained in the three fractions permit the calculation of the contribution of every one of the three fractions to the total difference in P/N ratio. As shown in TABLE 1, the qualitative difference in the first two fractions accounts only for somewhat over one half of the actual difference in the two strains; the remainder must be due to the larger amount of material in the fraction sedimented by 24,000 × g. in C57 as compared with BALB.

The difference in P/N ratio between the two strains turns out to be a complex character, involving at least two components: a qualitative difference in the particles thrown down by low centrifugal forces and a quantitative difference in the mitochondria sedimented by high centrifugal forces. This result is in complete agreement with the conclusion drawn from the immunological analysis that there must be a difference in at least two antigens, one of which involves an increased amount of a common substance in C57 mitochondria. An important conclusion to be drawn from these results is that different fractions of mitochondria may vary independently in different strains. In other words, they react in a different manner on the influence of the same genotype.

The difference in the liver mitochondria of the two strains can also be demonstrated morphologically. In collaboration with Miss Carol K. Williams, we

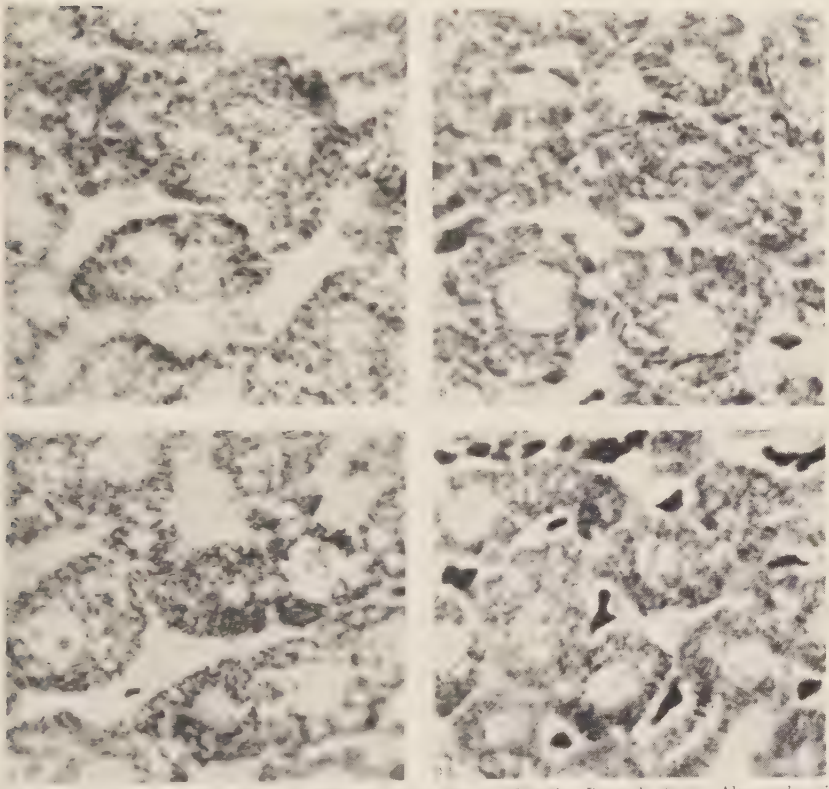

FIGURE 1. Liver mitochondria from different mouse strains. Fixation: Regaud. Stain: Altmann's aniline-fuchsin. Magnification: ×1075. (*a*) BALB male, (*b*) C57 male, (*c*) BALB female, (*d*) C57 female.

were able to show, in preparations fixed with Regaud and stained with Altmann's aniline fuchsin, that the liver mitochondria of C57 males can be distinguished from those of BALB males (FIGURES 1 and 2, *a* and *b*). The shape of individual mitochondria in a single cell is subject to a large amount of variation; but C57 male mitochondria are characterized by the prevalence of large thick rods and large granules, whereas long thin filamentous mitochondria, together with smaller granular forms are found predominantly in BALB male liver cells. The livers from the males of the two strains can be distinguished from each other with certainty by the shape of their mitochondria.

This does not apply to the females (FIGURES 1 and 2, *c* and *d*). The liver cells of females from both strains contain, predominantly, rodlets of medium length and thickness. They can be distinguished with certainty from C57 males, but the difference between the females of either strain compared to BALB males is not always clear. This observation is in agreement with the values for P/N ratio of isolated liver mitochondria from females of the two strains (Caspari, 1954). The values found were 98.30 ± 1.63 mg. P/gN for BALB females and 99.15 ± 1.63 mg. P/gN for C57 females, an insignificant difference. Furthermore, the P/N value of the mitochondria isolated from

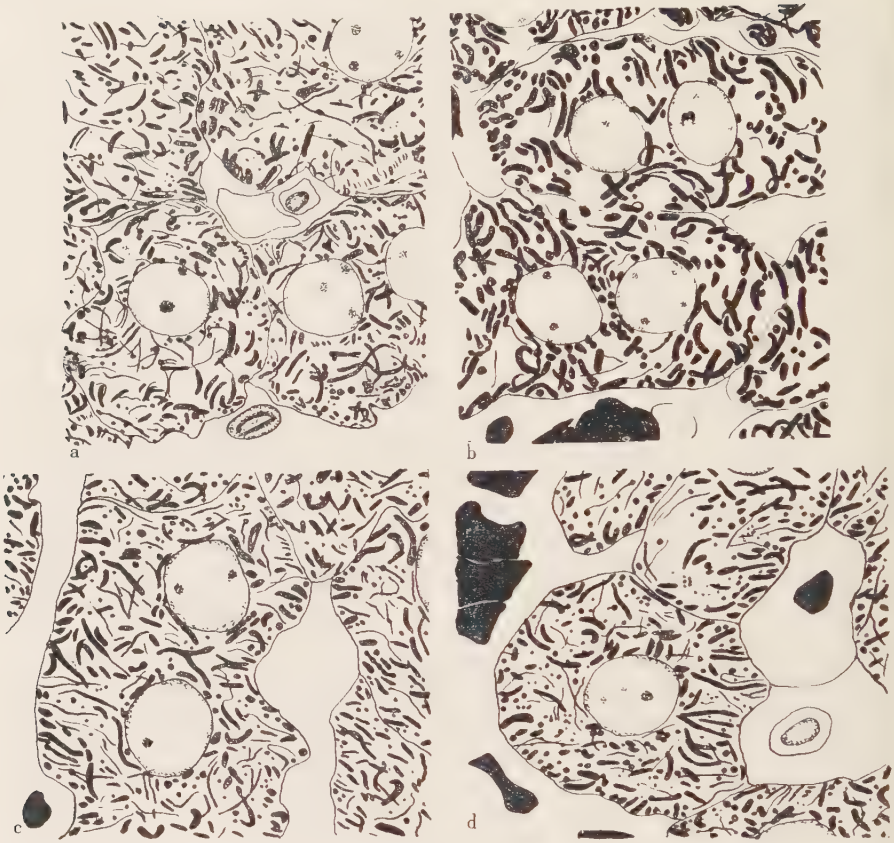

FIGURE 2. Mitochondria in liver cells from different mouse strains. Fixation Regaud. Stain: Altmann's anilin-fuchsin. Drawings by Carol K. Williams. (*a*) BALB male, (*b*) C57 male, (*c*) BALB female, (*d*) C57 female.

the livers of females is significantly different from that of C57 males, but not from BALB males.

In summary, it may be stated that the strain difference in mitochondrial structure has been demonstrated by three independent techniques: immunological, chemical, and cytological. These methods support each other insofar as both the immunological and the chemical method indicate that the difference between the two strains is a complex phenomenon involving at least two components, one of which is certainly quantitative. The chemical and cytological methods agree in indicating that the strain difference is restricted to the males and that, in C57, there exists a difference between the two sexes. It may be suggested that these pronounced differences between the mitochondria of the two strains may be accompanied by differences in their enzymatic functions. Actually, Lehninger and Kennedy (1948) have observed differences in sensitivity to malonate between the liver mitochondria from two different strains of rats.

TABLE 2

MEAN P/N RATIOS OF LIVER MITOCHONDRIA FROM MALES OF BALB, C57 Bl AND THEIR CROSSES MG. P PER G. N

Strain	P/N ratio ± SE	Number
BALB	96.26 ± 0.96	76
C57	102.03 ± 0.65	75
β F_1	99.62 ± 1.55	27
ζ F_1	98.45 ± 1.43	27
β F_2	98.60 ± 1.24	29
ζ F_2	102.53 ± 1.28	31

The Inheritance of the Mitochondrial Difference Between C57 *and BALB*

The chemical difference between the isolated liver mitochondria of the two mouse strains C57 and Bagg albino is large enough to permit a genetic analysis. The two strains were crossed, and the mitochondria from the livers of F_1 and F_2 animals isolated and the P/N ratios determined. The results for the males are shown in TABLE 2. The crosses were carried out in both reciprocal directions. In the table, a cross of BALB mother to a C57 father is indicated by the Greek letter β, the reciprocal cross by the letter ζ. The table shows that in F_1 no reciprocal differences are apparent, and that the P/N values appear to be intermediate between the two strains. The P/N ratio in F_2 approaches the value of the males from the grandmaternal strain.

This result is typical for cases of cytoplasmic inheritance (Caspari, 1948). In cytoplasmic inheritance, it is rare that a particular phenotype is dependent on the cytoplasm alone. In most cases, a phenotype is dependent on the collaboration of a particular cytoplasmic constitution with a particular set of genes. Genes of this type are called plasmon-sensitive genes. If a phenotypic character is dependent on a cytoplasm and one recessive plasmon-sensitive gene, no difference between the reciprocal crosses will be found in F_1, since the dominant allele will inhibit the phenotypic expression of the gene in the heterozygote. In F_2, the homozygous recessive will be reconstituted, and will produce the corresponding phenotype, but only in the appropriate cytoplasm, *i. e.* in one only of the two reciprocal crosses segregation for the character will be found. Cases of this kind, involving one gene, have been described for pollen-sterility in *Linum* by Bateson and Gairdner (1921), for a flower abnormality in *Epilobium* by Michaelis (1940), and for sex determination in some *Streptocarpus* crosses by Oehlkers (1941). In the majority of cases, however, more than one plasmon-sensitive gene is involved, as has been analyzed particularly by Michaelis (1954) in *Epilobium* crosses. The result of the crosses between C57 and BALB mice fits the interpretation that the difference in mitochondrial P/N ration is due to a cytoplasmic component in addition to several recessive plasmon-sensitive genes. The cytoplasmic component is expressed by the difference between reciprocal crosses in F_2. The genes controlling the phenotype are recognized by the lack of a reciprocal difference in F_1. In addition, there is evidence that the variance is larger in F_2 than in F_1 and in the original strains, indicating genic segregation in F_2.

TABLE 3

MEAN P/N RATIOS OF LIVER MITOCHONDRIA FROM FEMALES OF CROSSES BETWEEN BALB AND C57 B1 MG. P PER G. N

Cross	P/N ratio ± SE	Number
β F_1	91.07 ± 1.63	23
ζ F_1	92.57 ± 1.56	24
β F_2	90.22 ± 1.21	25
ζ F_2	91.76 ± 1.33	25

In the females, no difference between reciprocal crosses could be found in F_1 and F_2 (TABLE 3). This finding is not unexpected, in view of the fact that no difference between the females is found in the original strains. It has been argued in an earlier communication (Caspari, 1954) that the difference in the genetic behavior between females and males cannot be attributed to sex-linked genes, but is probably to be regarded as sex-controlled.

The genetic data indicate, therefore, that the P/N ratio of mitochondria of the two mouse strains studied is under control of nuclear genes. A particular phenotype, however, does not depend on the genes alone, but also on a maternally transmitted cytoplasmic factor. This factor could be assumed to be the self-reproducing mitochondria themselves. The finding may not be used, however, as an argument in favor of the self-reproduction of these structures, since it can be well imagined that the cytoplasmic factor transmitted is a precursor of the mitochondria, or a self-perpetuating condition influencing their chemical constitution. It should be pointed out that the results described bear a great similarity to the genetic behavior of plastids in plants which are also dependent in their differentiation on genes, but which, at the same time, have maternally transmitted properties of their own which determine their reaction to a particular genotype. A similar situation holds for the mitochondria of yeast according to the work of Ephrussi and his collaborators (1953), in which inactivation of the mitochondria can be produced by a genic mutation or by a direct effect on the mitochondria.

Influence of Individual Genes on Mitochondria

The strains C57 and BALB are distinguished by a large number of genes. It is, therefore, difficult to ascribe the differences in mitochondrial composition between the two strains to any particular one of these genes. The influence of individual genes on the liver mitochondria was therefore studied in a different genetic system.

The two genes T (Brachyury) and Fu (Fused) in the mouse were chosen for this investigation. Both are dominant genes affecting phenotypically the length and structure of the tail. The first one is lethal in homozygous condition, the other viable. Both are situated in the same chromosome and are closely linked (Dunn and Caspari, 1945).

Both genes had been outcrossed to the Bagg albino strain for over 20 generations. The result is that they carried the same genes as the BALB strain, except for the mutant gene in question and a small chromosome piece surround-

ing it which may not yet have become isogenic by crossing-over. In the case of the T strain, it could be shown by tumor transplantations that the strain carried the same allele of the heterocompatibility gene H-2d as the strain BALB even though the H-2 locus is closely linked to the two tail mutants (Snell and Higgins, 1951). Both strains, therefore, carry the same genetic complement and the same cytoplasm as BALB, and are different only by the genes T and Fu, respectively, and possibly a small chromosome piece surrounding them.

Liver mitochondria from the three strains were isolated, and rabbits immunized against these preparations. Mitochondrial antigens from the different strains, adjusted to contain 0.04 mg. N/ml., were compared by means of the quantitative complement fixation method, as indicated before. In all experiments, male mice were used.

In comparing the reaction of T/+ and +/+ liver mitochondria to anti-T/+ sera, it was found that the T/+ antigen reacted at a higher titer than the +/+ antigen. This reaction indicates clearly the existence of an immunological difference between the two strains. As far as Fu/+ liver mitochondria are concerned, it turned out, in titrations for anticomplementary activity incidental to complement fixation tests, that they showed consistently a higher titer, relative to the same amount of nitrogen, than +/+ and T/+ mitochondria. This finding indicates that Fu/+ liver mitochondria must be in some way different from T/+ and +/+ particles. In other words, it is possible, by means of titration for anticomplementary activity and, by the use of specific antisera, to distinguish liver mitochondria from +/+, T/+, and Fu/+ animals. These effects must be due to differences induced by the genes T, Fu and their normal alleles, or possibly to genes very closely linked to them. It should be emphasized that, morphologically, the main effects of these genes are on the tail structure, and that this result indicates that other organs which are not affected morphologically may still show the effect of these genes by their antigenic constitution. At all events, the evidence seems to indicate that even small genetic differences may lead to demonstrable differences in mitochondrial constitution.

Tissue Differences in Mitochondrial Constitution

Relatively little work has been done on mitochondria from organs other than liver (Schneider, 1953). It appears from the literature that particulate fractions isolated from organs other than liver show the same general properties but minor differences in chemical composition and enzymatic activity. In order to determine whether the genic influence on mitochondria is restricted to liver only, mitochondria from C57 and BALB kidneys were investigated for their P/N ratio. It should be pointed out in this connection that, while liver tissue consists predominantly of one single type of cell, the kidney consists of several different types of cells, and the isolated mitochondria from kidney may be presumed to constitute a mixture derived from all these different types of cells.

In 12 males, 6 BALB and 6 C57, mitochondria from both liver and kidneys of the same individual were isolated and the P/N ratio determined. It turned

TABLE 4

ANALYSIS OF VARIANCE OF ISOLATED MITOCHONDRIA FROM C57 AND BALB HOUSE KIDNEYS

	Sum of squares	Difference	Estimate of variance
Between experiments	4,876.09	8	609.51
Between strains	391.61	1	391.61
Interaction	1,283.77	8	160.47
Residual	2,181.76	34	64.17
Total	8,733.23	51	

out that kidney mitochondria had a slightly higher P/N ratio than liver mitochondria. The difference, 11.06 ± 3.725 mg. P/gN is significant at the 2 per cent level. It may be suggested that this difference in mitochondrial composition in the two tissues is related to differences in the function they exert in these two organs.

Mitochondria isolated from 26 BALB and 26 C57 male kidneys were investigated for the occurrence of a strain difference. The difference, 5.48 ± 3.578 mg. P/gN is large, but not statistically significant ($t = 1.53$, $df = 50$, $P > .1$). The data were derived from nine individual experiments in which an equal number of C57 and BALB animals were used. In three of them, BALB had the higher P/N ratio, in the remaining five, C57. An analysis of variance gives, however, evidence for a strain difference in the kidney mitochondria (TABLE 4). While the variance due to experiments is highly significant ($F = 9.50$ for 8 and 34 df, $P < .01$), the variances due to strains and due to interaction between strains and experiments are significant at the 5 per cent level ($F = 6.10$ for 1 and 34 df, $P < .05$, and $F = 2.50$ for 8 and 34 df, $P < .05$ respectively). The latter observation suggests that the kidney mitochondria of the two strains react differently on the factors responsible for the variability between experiments.

At all events, it becomes clear that the mitochondria are very labile structures whose composition is influenced by a variety of factors. Among the factors we have been able to identify are genes, sex, and the tissues from which the particles are derived. In addition, it has been shown that environmental factors such as diet, starvation, cold, and drugs influence the morphological appearance of the mitochondria profoundly (Steffens, 1941; Roberts, 1949; Kater, 1933). Beyond this variability, there have been demonstrated cytoplasmically controlled properties of the mitochondria which are transmitted unchanged through at least two generations, and which control their reaction to genes.

Discussion

The developmental problem can now be posed in a more precise way. Cytoplasmic particles are influenced by genes. At the same time, they show different characteristics in different types of cells carrying presumably the same genes. This difference must be the result of a developmental process, and constitutes a part of cellular differentiation. It may be asked, therefore, how differ-

ent types of cells acquire a different set of cytoplasmic particles in the presence of identical genes, and how, on the other hand, specific genes affect the differentiation of cytoplasmic particles in the same type of cell.

One possibility which may be suggested is differential distribution of different mitochondria to the daughter cells at mitosis. Unequal cell division may certainly be identified as a mechanism of differentiation, as is clearly shown for the determination of scales in Lepidoptera (Henke, 1948) and of bristles in *Drosophila*. It can well be imagined that, in the process of unequal cell division, different types of mitochondria or their precursors are distributed unequally to different cells. These mitochondria would then determine the type of metabolism of the cell and the differentiation it would undergo. The differential reaction of different mitochondria to the same genotype would explain the "pattern of manifestation" (Hadorn, 1945) characteristic for the effects of mutant genes.

Unequal cell division, however, cannot be regarded as the only means, or even as the principal means of differentiation. The principles developed by Gustafson, (1953) for the sea-urchin egg may be of wider application. In the sea urchin, an animal-vegetal gradient of mitochondrial development appears in the mesenchyme blastula. The gradient is correlated with the original double gradient system of the sea-urchin egg, and is expressed particularly by a mitochondria inhibiting influence of the vegetal type of metabolism. This concept is borne out by the observation of larvae vegetalized by Li, animalized by iodosobenzoic acid, and by the observation of animal and vegetal fragments (Gustafson and Lenicque, 1953). A parallelism between the visible appearance of mitochondria and the rate of increase in certain enzyme systems makes it probable that these enzymes are connected with the development of the mitochondria. Gustafson, in turn, suggests that the metabolic activities of the mitochondria are instrumental in the formation of the typical differentiations of the animal cells, stretching of the cells, and the formation of cilia.

In this case, the appearance of the cytoplasmic particles marks the first step in the course of differentiation. Their appearance is regulated by the position of the cell in a gradient field or, for later processes, by the relation of the cell to other developing areas. It may be assumed that different types of cytoplasmic structures are formed, depending on the type and position of the cell.

Every cell seems to possess the potential ability to form different types of cytoplasmic particles. At least some of them seem to be of more or less general occurrence through all types of organisms. Structures similar in enzymatic activity and composition to the mitochondria of mammalian liver have been shown to exist in yeast (Ephrussi, 1953), echinoderm larvae (Gustafson, 1952), plants (Bonner and Millerd, 1953), and bacteria (Mudd, 1953). In any particular type of cells, several types of particles with different functions and properties may be found, the composition of the particle population being characteristic for the cell type. This composition would confer on the cell a characteristic metabolic pattern which is expressed by its developmental and physiological activity.

The characteristics of the cytoplasmic particles are, to a certain degree, determined by the cytoplasm itself, as demonstrated by the cytoplasmic inheri-

tance of the P, N ratio of liver mitochondria. There is, in addition, a definite genic control, so that the final phenotype of the cytoplasmic particles is the resultant of the genic and the cytoplasmic component. The genic influence may be of different types. It may change the quantity of a specific type of particle, such as the small mitochondria in C57 and BALB mice livers, or it may change the quality of a specific type of particle, as shown for the large mitochondria of C57 and BALB mice.

The similarity of differentiated cells in different species of animals can be ascribed, therefore, to their fundamentally similar potentialities for the formation of cytoplasmic particles. Genes appear to be responsible for the actual composition and the properties of the particles formed.

Summary

Differences between the liver mitochondria of the mouse strains of C57 Bl and BALB have been demonstrated by means of immunological, chemical, and cytological methods. Both the chemical and the serological investigations show that the strain difference is a complex character involving at least two components. The mitochondria constitute a population of particles with different characteristics which react differently on the action of the same genotype. Crosses between the two strains show no difference between reciprocal crosses in F_1, but significant reciprocal differences in the maternal direction in F_2. It is concluded that the characteristic biochemical composition of the two strains is dependent on the interaction of several recessive genes with a maternally inherited cytoplasmic factor. The individual genes T and Fu influence the antigenic structure of the liver mitochondria. Kidney mitochondria have a slightly, but significantly, different composition from liver mitochondria derived from the same animals. The existence of a strain difference in the composition of kidney mitochondria has been demonstrated. Every cell possesses the potency to form different types of cytoplasmic particles. The differential particle patterns characteristic for different cell types may arise through unequal cell division or, more frequently, through other determining influences such as embryonic gradient fields. Genes are involved in the control of the quality, differentiation, and quantity of the cytoplasmic particles.

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DEVELOPMENT IN THE PRESENCE OF ANTIBODIES*

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A suggestion originating with Doctor H. Clark Dalton that mental and operational methodology should be emphasized at the expense of presenting detailed results was a fortunate one in connection with this paper because, in reviewing the work on development in the presence of antibodies, one finds that, although many preliminary reports have been made, few if any definitive results have been recorded.

The atmosphere of this monograph is charged with examples emphasizing the significance of proteins or other high molecular weight compounds in cellular physiology and in developmental mechanics. It would be redundant to cite further examples here, many of which can be found in the reviews of chemical embryology by Needham (1931, 1942) and Brachet (1950a). Pertinent to this discussion are the now well-known hypotheses of Tyler (1947) and of Weiss (1947, 1953a) which relate numerous aspects of developmental phenomena to highly specific molecular interactions that depend upon the extraordinary complexity demonstrated by proteins and other large molecules. Similar ideas have been presented by Rose (1952a, b, 1955), who suggested a mass action relationship to explain successive steps of differentiation and size regulation. In support of these ideas, Rose has presented experimental observations in which the addition of pieces of adult frog organs to culture media suppress the development of homologous organs in the embryo. It seems clear from his arguments that he visualized proteins as the major participants in this mass action. Weiss and his co-workers (1952, 1953a), in further elaboration of his earlier hypotheses, which depend on concepts of antigen-antibody type molecular interactions, found that the presence of embryonic heart or kidney extract in chick-organ cultures reduced the differentiation capacity of the homologous organ. In another experiment, the intravenous injection of embryonic kidney brei led to increased mitotic count. Weiss placed the emphasis on an antagonism between growth and differentiation. More recently, Ebert (1954) has presented evidence for alternate explanations of these observations.

While the conflicting evidence prevents a critical analysis of these reports, it seems possible that species and stage differences have influenced their results. Consequently, a detailed description of the chemical, presumably protein, composition and interactions in these species at different stages becomes necessary.

Thus, with a massive literature pointing to the unique position of proteins, embryologists are forced to search for experimental approaches which will throw light on their role. These methods must be applicable to studies on the earliest developmental stages and must not only identify the complex molecular species involved, but must also suggest their functions. It would seem

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profitable to examine briefly several methods of analysis from the point of view of their contribution to this question.

The methods of modern biochemistry have contributed immeasurably to our understanding of energy relationships and pathways of intermediary metabolism and have provided some powerful analytical procedures. However, out of 909 pages in a recent biochemistry textbook (Fruton and Simmonds, 1953), only 50 are devoted to the properties and functions of intact protein molecules other than the well-known enzymes and protein hormones which will be discussed below. Under a title suggesting a discussion of proteins, one finds, instead, a discussion of their constituent amino acids. Those who have attempted to consider the amino acids as an approach to an understanding of development have invariably encountered difficult problems of interpretation. As representative of these studies, Holtfreter *et al.* (1950) indicated that the amino acids show neither quantitative nor qualitative changes throughout development, and thus one cannot directly relate them to specific developmental events. While Kutsky *et al.* (1953) and Kavanau (1953, 1954) have found quantitative fluctuations in amino acids during development, their major conclusion was that proteins do change during development. Herrmann (1953), in a study utilizing amino acid analogues to interfere with normal development, observed that different analogues produced qualitatively different effects and postulated that they inhibit the formation of different proteins to a different extent, but he could not identify these proteins specifically. In other studies, utilizing viscosimetric methods, Herrmann (1952) was able to relate quantitative changes in muscle proteins to muscle morphogenesis, though the earliest stages tested were well after the initial organogenesis. It must be noted that this test is a rare instance in which the protein under consideration has been well characterized biochemically, thus permitting rather precise measurement. Other instances of this nature can be cited: *e.g.*, the studies on foetal hemoglobin (Roughton and Kendrew, 1949) and the electrophoretic studies of embryonic blood (Moore *et al.*, 1945; Marshall and Deutsch, 1950); but it seems unlikely that a sufficient number of proteins will be adequately characterized to make these techniques useful for many problems in the near future. In addition, these examples are limited to the analysis of proteins, which form the major component of the organ or tissue in question. Yet, as will be discussed below, it is probable that the minor components may be of greater developmental interest (*cf.* Weiss, 1947, 1953a).

The protein hormones come to mind as materials for which testing procedures are available and, indeed, their study has proved profitable in many lines of developmental studies. Ample evidence, however, points to the fact that most hormones exhibit their effect relatively late in development (Willier, 1952). Weiss (1939a, p. 442) provides a fundamental reason why studies of hormones will not be productive of answers at the early stages; namely, that "diffusing agents could never produce manifest differences among parts unless the parts were already latently distinguished from one another." Thus, experimental and logical reasons lead us in other directions in our search for an understanding of the part played by proteins in the processes of early development.

Perhaps the most thoroughly investigated field representing the part played

by proteins has been the study of enzymes in development (*cf.* Needham, 1931, 1942; Brachet, 1950a; Flexner, 1955). Frequently these studies have been concerned with phenomena not directly related to the question of the protein enzymes concerned, but to their activities in the provision of energy, or for the analysis of other metabolic events. On the other hand, very suggestive studies have been directed toward the problem of the time and place of enzyme appearance, utilizing the enzymatic activity as an indication of their presence (*cf.* Moog, 1952). Shen (1955), in a recent comprehensive review of the problems of enzymatic studies on the embryo, has pointed out some of the difficulties inherent in their interpretation.

One handicap in these studies, which must be mentioned here, is that only those enzymes for which a function is known can be studied. Because of this difficulty, enzymatic analysis of the embryo tends to lag behind the work of the enzymologists who utilize more favorable materials. Thus, these studies may or may not have a direct pertinence to morphogenetic problems of protein development, depending upon the fortunate choice of the investigator. More often, as has frequently been mentioned, the analysis becomes a study of the end point of a process of chemogenesis, which, in itself, is of interest, but does not tell us the story about the development of a function prior to the time it becomes functional.

Thus, we must conclude that these techniques, which frequently depend on the normal functions of the proteins, cannot be used with the greatest hope of answering our questions. What method of analysis, then, is needed? First, it must be highly sensitive and highly specific; second, it must be independent of the normal functions of the material being tested.

The specificity must be sufficient to distinguish between highly similar materials present in exceedingly small amounts. This quantitative and qualitative specificity is provided by the serological reactions, as has been amply attested by Landsteiner (1946) in his classical treatise and by many other investigators. The quantitative techniques introduced by Heidelberger and Kendall (1929) and the qualitative antisera-agar techniques introduced by Oudin (1946) and Ouchterlony (1948), together with the older techniques (Boyd, 1947; Kabat and Mayer, 1948; Raffel, 1953) have made this tool as precise as any to be found (*cf.* the recent review by Corcoran, 1952).

Utilizing serological tools, the first task was to show that the embryo does, indeed, contain antigens. This task has been adequately reviewed by Cooper (1946), Schechtman (1947, 1955a), Ten Cate and Von Doorenmaalen (1950), Woerdeman (1953b) and others, with the overwhelming decision in favor of the antigenicity of the embryo. The second, and still the major task, has been that of the explorer: to discover the antigens, to plot their distribution and changes in time and space, to measure and identify them, and, if possible, to suggest their functional relationships. This activity has gathered momentum, as shown in TABLE 1, in which some of the reports which have been published using *in vitro* techniques with nonliving materials have been tabulated. These studies show that certain antigens are common to all stages of development; that some are first detected during development and may be related to morphogentic events or the appearance of organ and species differentials (Loeb, 1945);

TABLE 1
SOME IN VITRO STUDIES OF EMBRYONIC ANTIGENS SINCE 1935

Author	Animal	Preparation	Test
Perlmann & Gustafson, 1948	Sea urchin	Saline soluble homogenates	Precipitin
Perlmann, 1953	Sea urchin	Saline soluble homogenates	Antisera-agar
Harding *et al.*, 1954	Sea urchin	Saline soluble homogenates	Antisera-agar
Averch & Heronimus, 1937	Bee	Saline soluble homogenates	Precipitin
Telfer & Williams, 1950, 1951, 1953	Silkworm	Blood & yolk	Antisera-agar
Telfer, 1953, 1954	Silkworm	Blood & yolk	Antisera-agar
Burke *et al.*, 1944	Frog	Organ extracts	Precipitin
Ten Cate & Van Doorenmaalen, 1950	Frog	Organ extracts	Precipitin
Cooper, 1946, 1948	Frog	Homogenates & blood	Precipitin
Cooper, 1950	Frog	Homogenates & blood	Antisera-agar
Spar, 1951, 1953	Frog	Homogenates & blood	Antisera-agar
Flickinger & Nace, 1952	Frog	Homogenates & blood	Antisera-agar, p'tin
Woerdeman, 1950, 1953	Axolotl	Homogenates, embryonic regions	Precipitin
Clayton, 1951, 1953	*Triturus*	Homogenates, embryonic regions	Precipitin
Burke *et al.*, 1944	Chicken	Organ extracts	P'tin, complement fix.
Schechtman, 1947, 1948	Chicken	Organ extracts	Precipitin
Ebert, 1949, 1950, 1951, 1952, 1953, 1954, 1955	Chicken	Organ extracts	Precipitin
Ten Cate & Van Doorenmaalen, 1950	Chicken	Organ extracts	Precipitin
Schechtman, 1947, 1952, 1955	Chicken	Blood, yolk & fractions of	Precipitin
Nace & Schechtman, 1948	Chicken	Blood, yolk & fractions of	Precipitin
Schechtman & Nace, 1950	Chicken	Blood, yolk & fractions of	Precipitin
Marshall & Deutsch, 1950, 1951	Chicken	Blood, yolk & fractions of	Precipitin
Schechtman & Hoffman, 1952	Chicken	Blood, yolk & fractions of	Precipitin
Schjeide, 1952	Chicken	Blood, yolk & fractions of	Precipitin
Nace, 1953	Chicken	Blood, yolk & fractions of	Precipitin
Darrow *et al.*, 1940	Mammal	Hemoglobin	Precipitin
Stratton, 1943	Mammal	Blood group antigens	Agglutin. & lysis
Maculla, 1948	Mammal	Organ extract	Complement fixation

and that still others disappear during development. Some studies; *e.g.*, Ten Cate and Von Doorenmaalen (1950) for frog lens, and Ebert (1953, 1955a) and his co-workers (1955b) for chick heart, have clearly demonstrated that specific antigenic changes occur in organ-forming areas prior to histological differentiation. The very interesting study of Harding, Harding, and Perlmann (1954), taking advantage of antigenic differences between *Paracentrotus*, *Psammechinus*, *Arbacia*, and their hybrids, has made a marked contribution to our information on the time of participation of the sperm in development. While much of the evidence (TABLE 1) points in the direction of qualitative changes following these patterns, it is more certain that quantitative changes occur. Thus, these results resemble those derived from studies of enzymatic changes in development.

From such *in vitro* studies, it is seen that a great deal can be suggested as to the functions of antigens in development (*cf.* Nace, 1953; Schechtman, 1955a). Any suggestion made in this respect, however, must remain largely inferential, because, usually, only a time correlation between the appearance of an antigen and the concomitant appearance of a physiological or developmental function can be made. For critical conclusions, a direct test must be applied; *e.g.*, the manipulation of a specific protein must lead to the modification of a specific developmental event (*cf.* Harding *et al.*, 1954). Woerdeman's report (1953a, b) of obtaining serologically specific axolotl lens antigen by *in vitro* incubation of a mixture of presumptive lens ectoderm and eye vesicle extracts is an example of such an experiment.

Another shortcoming in these studies has been that much of our information has come through the analysis of well-known protein systems (*cf.* ten Cate and von Doorenmaalen, 1950; Ebert, 1953; Nace, 1953) and suffers from the same handicap that is experienced in the enzyme studies. It carries our knowledge from the adult level back to the embryonic level, which, important though this is, does not suggest the morphogenetic significance of protein systems limited to the embryonic period, or of only minor importance to the adult, or which, though functional in the adult, have not yet been studied. The basic assumption behind much of the serological work listed in TABLE 1 has been that such unknown but important antigenic systems do exist. Evidence for their existence is found in the reports of "new antigens" or "embryonic antigens" demonstrated after appropriate absorption procedures and, even more suggestive, the numbers of precipitation bands observed in the studies utilizing the antisera-agar techniques. These bands probably represent antigens of which we have no other knowledge. Besides this serological evidence, the few electrophoretic (Moore *et al.*, 1945; Marshall and Deutsch, 1950, 1951; Flickinger and Nace, 1952) and ultracentrifugal (*cf.* Moore *et al.*, 1945; Marshall and Deutsch, 1950; Schjeide and Deutsch, 1953) studies which have been made on the embryo, indicate the presence of high molecular weight materials of unknown nature and function. It is likely that further studies will demonstrate the presence of many less abundant materials which, however, must have significant functions.

These considerations suggest that investigations utilizing antisera produced in response to antigens obtained from the embryo, technically difficult though

this may be, may prove highly fruitful. At the same time, one must not lose sight of the possible morphogenetic significance of antigens derived from the maternal organism (*cf.* reviews by Brambell *et al.*, 1951; Schechtman, 1955a).

In spite of this last point, the above argument challenges the continuing value of the criteria suggested by Ebert (1953, p. 343) in the selection of antigenic systems for study. Ebert asks whether or not it will be possible to characterize nonheart-organ-forming areas by immunochemical or biochemical methods and sets the following criteria for selection of an organ or tissue for successful experimentation in this direction: (1) "it must be an organ which attains early morphological or functional status"; and (2) "for which a sound chemical basis of function has been established."

There is no dearth of organs or tissues which meet the first criterion. From the organelles of the prefertilized egg on through development, specific regions can and have been assigned morphological or functional values; *e.g.*, the notochord. It is precisely the second criterion which forms the basis for the major question we have to ask: "What is the chemical basis of function of morphogenetic areas?"

Though physical phenomena may be immediately responsible for many of the events in morphogenesis (*cf.* Weiss, 1939a; Holtfreter, 1943), it seems certain that these conditions must refer back to chemical interactions (Ranzi, 1951, 1953). Thus, the task of the embryologist must be to elucidate those relationships that are, in all likelihood, either nonexistent or rare (perhaps pathological) in the adult in which our usual "sound chemical basis of function" is established.

We must ask: "How is it possible to establish the chemical basis for a function when the function is unknown or poorly understood?" Perhaps it is in the implications of the question asked by Sigurdsson (1940): "What components of the cell are so vitally important that their blocking by antibodies is incompatible with the growth and proliferation of the cell?" Here we see the suggestion of a guide to significant components and their activity, detected by a means independent of the normal function of the material.

This suggestion brings us to the second criterion for a method of analysis, the first referring to specificity, the second referring to independence of the normal functions of the material being tested.

Again, we turn to the serological technique. That this method is independent of the normal biological functions of the materials being tested is inherent in the nature of the reaction. Thus, in the concept of serological reactions as developed by Breinl and Haurowitz (1930), Alexander (1932), Mudd (1932) and Pauling (1940), it is the unique reactive groupings on the surface of the antigenic molecule, either protein or other high molecular weight material, which stimulate the production of specific reagents by the antibody producer and which then combine with the antisera. If, by chance, these sites are responsible for the biological activity of the antigen, then that activity may be blocked by the combination of antigen and antibody. This phenomenon seems to occur in the case of lecithinase, which is inhibited by antivenin (Morgenroth and Kaya, 1910). On the other hand, Landsteiner (1946, p. 38) reviews many instances in which the activity of hormones and enzymes is not inhibited by anti-

sera. Thus, the serological reaction is a test that does not depend upon the normal biological activity.

In spite of this independence, it has been found, in many instances, that exposure of living cells to appropriate antisera interferes with their function and viability, a fact which has given rise to the term "cytotoxins" (*cf.* reviews in Landsteiner, 1946, p. 82; Raffel, 1953, p. 468). It is difficult to know the precise mechanism of this interference. In some cases, it may be by agglutination of cells or by weakening their membranes, as in lysis, or otherwise affecting the structural integrity of the cell or its organelles (*cf.* Irwin, 1949; Grunwalt, 1949). These visible effects may be caused by the combination of antigen and antisera which, by reducing solubility or restricting free motion, or by changing essential physical and chemical characteristics of the molecule, effectively remove the antigen from its site of normal function. These conditions would not inhibit enzymatic reactions *in vitro*, because of the freedom from the restricting conditions of the cell. Thus, it could be expected that antisera might show toxic effects on embryonic material exposed to them.

On the other hand, and more difficult of explanation, are the few poorly supported reports of a stimulating effect of antisera. Asai and Umeda (1929) reported that rabbit antisera directed against the saline soluble supernatant of *Rana esculenta* pharangeal ciliated epithelium accelerated ciliary activity when placed on isolates of such an epithelium. Parallel tests with normal rabbit serum, taken before injection of each rabbit, did not produce this effect. These investigators offered no suggestion as to the mechanism of this response. Several possibilities exist, however: (1) the cilia may have been accelerated as a "final act" of the cells prior to succombing to a cytotoxic agent; (2) the antisera may have inactivated an inhibiting agent, thus releasing the ciliary mechanism; (3) the antisera may, by coincidence, have been directed against "templates" which produced an enzyme essential to the ciliary mechanism, and this antibody may therefore have resembled the enzyme configuration closely enough to act as supplemental enzyme. As the first possibility seems the most likely, this effect may be only an apparent case of stimulation by antibody.

Weiss (1947) reported a relative stimulation of growth of the homologous embryonic organ on *in vivo* exposure to antiorgan sera and suggested a "template" mechanism to explain these observations. Further analysis (Weiss, 1953b) has related this organ enlargement to vascular damage and resultant hemorrhages, thus throwing serious doubt on the growth-stimulating action of the antisera.

Other reports of possible "stimulating action" of antisera are the early descriptions by Metchnikoff (1899) of a stimulation effect by homologous antisera at low dosage and the suggestion by Bogomolets (1943) of the stimulating effect of certain antisera on the reticuloendothelial system. Pomerat and his co-workers (*e.g.* 1945, 1949), in a long series of experiments, have not been able to confirm the results of either of these workers. Another instance is the observation by Goldfeder (1953) of an apparently stimulating effect on Sarcoma 180 of normal tissue implants from mice which had been "immunized" against this tumor. Here, the serological nature of the response has not been confirmed. The activation of sea-urchin eggs by antisera reported by Perlmann

(1954) could be interpreted as either stimulating or sublethal cytotoxic action. Finally, if we did not know the nature of the mechanism, observations of the response to the "protective" action of antitoxins, *etc.*, might have been interpreted as a stimulating action of the antisera.

Thus, while it need not necessarily be expected that living systems exposed to antisera will respond with characteristic symptoms of cytotoxic action, stimulating effects of antisera have not been established. Therefore, though this possibility must be kept in mind, the following discussion will concern itself with cytotoxic effects.

Exploitation of the cytotoxic action of antisera has constituted a Holy Grail that has eluded the efforts of a great army of cancer investigators. The idea originated in the early days of immunology (reviewed in Grunwalt, 1949), when it was felt that there might be a possibility, growing out of the new and exciting advances in the field of immunity to infectious diseases, that the development of abnormal tissues might be prevented by the use of antibodies showing specific cytotoxic character. The problem here, as in embryology, was to characterize a unique function that was still unknown, and the question was asked: "What chemical basis for function of cancer tissue exists which, by its uniqueness, can be attacked and exploited therapeutically?" Usually the approach has been to attack and exploit without much serious question of the chemical basis. For example, a review of the papers by Lumsden, who was very active in this field from 1925 to 1937, does not reveal a single experiment in which an effort was made to isolate the antigen(s) responsible for the production of his "anticancer sera" (*cf.* Lumsden, 1925, 1937). It is not surprising that a controversy arose relative to the specificity of these sera (Phelps, 1937), inasmuch as a rational procedure hardly seems possible without some idea of the nature of the antigens involved. In more recent work, efforts are being made in this direction (*cf.* Grunwalt, 1949), and Nungester and Fisher (1954) report the production of a protective antiserum to one or more antigens found in the pellet produced by high-speed centrifugation of 6C3HED lymphosarcoma of mice whose genetic constitution was carefully controlled. These antisera seem to have highly specific action on specific cells. Similar considerations are involved in studies of the "immunity" to grafted tissues (Medawar, 1948; Billingham *et al.*, 1953; Ciba Symposium, 1954). In this case, however, the purpose was to avoid cytotoxic action. These experiences, which have been recently reviewed by Eichwald (1953), contain suggestions of value in the application of serological techniques to embryological problems.

The original search for antitumor sera utilized *in toto* animals as assay objects (Metchnikoff, 1899) and specific cytotoxic effects could not be differentiated from "general systemic effects." This finding led Lambert and Hanes (1911) to utilization of the tissue culture technique which was being developed at that time by Ross G. Harrison for certain embryological problems. Utilizing this technique, it is not surprising that early workers soon became interested in the action of antisera on embryonic cells, and thus contributed some of the first information on this subject.

In view of the possibility of obtaining specific cytotoxic sera (*cf.* Nungester and Fisher, 1954; Pressman and Keighley, 1948), one might ask: "Using such

sera at either lethal or sublethal doses, would it be possible to identify embryonic antigens of unique function, to localize them, and to manipulate them experimentally in order to analyze their developmental significance?"

Briefly, the idea would be to obtain specific antisera with cytotoxic action on the embryo or on embryonic explants, and to attempt to identify the specific antigens responsible for the cytotoxic action. This step would include the continuing dual approach of improving antisera specificity by appropriate absorption techniques and by injection of antigenic mixtures and fractions containing the active antigen in greatest concentration. Further attempts should determine the sublethal cytotoxic doses which, by modifying the normal function of the antigen, would lead to characteristic abnormalities that would be a key to the localization of the antigen and serve to suggest the time and nature of its action. Ebert (1950) has led the way in making use of this method in embryological studies. It must be noted, however, that lethal cytotoxic action may provide significant information along these lines. Death is usually not a "one-step phenomenon" and, as we shall see, observation of the progress of death may provide useful information. One wonders, therefore, about the embryos discarded by Clayton (1951, 1953) because of their death.

Further steps in the localization of these antigens require the utilization of the serohistological techniques developed by Coons *et al.* (1950) and by Pressman *et al.* (1948, 1950). Coons exposes tissues to specific antisera conjugated with fluorescein and observes the cellular localization of the antigen-antibody combination under suitable optic conditions, while Pressman utilizes antisera conjugated with I^{131} and detects the binding sites on tissue sections by autoradiographic techniques.

These biological criteria, combined with the methods of analyzing complex antigentic mixtures (Oudin, 1948; Ouchterloney, 1948), would necessarily be accompanied by suitable fractionation techniques such as salt and cold alcohol precipitation (Cohn *et al.*, 1946; Neurath and Bailey, 1953); the various electrophoretic methods (Longsworth, 1952; Henley, 1953); and, perhaps, ion exchange systems (Isliker, 1953), with the separations applied both to the antigens and the antisera. Such analysis could lead to a rationale for the application of the techniques of biochemistry and serology (Cohn, 1954) in a study of the metabolic histories of these embryonic components, many of which, by their unknown nature and presence in small quantities, would otherwise be impossible to detect.

Armed with this information, which could be available to us by the use of current techniques, it is possible to visualize the controlled experimental modification of embryonic physiology in the search for a deeper understanding of developmental mechanisms at a molecular level.

That this hope is not visionary is implied in the success of the bacteriologists and microbiologists in describing the antigenic and chemical composition of their organisms (Raffel, 1953, p. 430); in controlling and modifying their development (Spiegelman, 1948; Hardwick and Foster, 1952), as in bacterial transformations (Raffel, 1953, p. 422); and, especially, in the work of Sonneborn (1949) who, by the use of *in vivo* serological methods, has caused the transformation of *Paramecium* types.

Where do we now stand in our approach to the embryo from this direction? As discussed above, those investigating the cytotoxins in the search for methods of cancer diagnosis and cure have contributed certain information. The tissue transplantation group has also made a contribution in their effort to discover the rationale for successful tissue grafting (Eichwald, 1953) and have emphasized the genetic parameters of the problem (Snell, 1953; Hauschka, 1953). Further understanding of the pertinent genetic and nuclear-cytoplasmic relationships comes from blood group studies (*cf.* Irvin, 1949) and from other considerations (*cf.* Caspari, 1954; Schechtman, 1955b).

Direct studies on the embryo are still relatively few in number, as is seen from TABLE 2, in which some of the investigations utilizing *in vivo* methods are listed. This table does not include reports of embryonic development after the transmission of antibody from mother to young through the placenta or yolk (*cf.* Briles, 1948; Seegal and Loeb, 1940; Guyer and Smith, 1924), which have been previously reviewed (*cf.* Brambell *et al.*, 1951; Woerdeman, 1953b; Schechtman, 1955a), and the itemization of techniques and observations does not purport to be complete, but it suffices to demonstrate that this work has been scattered, directed toward many objectives, and far from uniform in approach.

The trend of these observations has been to show growth inhibition, appearance of developmental abnormalities, and loss of function followed by cellular damage and death. In general, the results are consistent with the *in vitro* studies in showing antigenic changes in the constitution of the organism during development. However, great differences are shown in detail, so that interpretation of antigen localization or function becomes difficult or impossible. It is believed that the primary reason for these discrepancies has been that the experimental design was not directed toward such analysis. Where specific contradictions are noted, the explanation probably lies in the nonuniformity of the reagents used. Antibody producers vary from individual to individual (Landsteiner, 1946) and, although the experiments may have superficial similarities, there is no doubt that each has dealt with different antigenic systems. It is evident that this method has not been exploited to its fullest capacity. The paucity of experiments since the development of more precise immunochemical techniques bears testimony to this lack of development. Only in the work of Ebert *et al.*, (1955), do we find a partial realization of this potential.

In this atmosphere of incomplete data, the discussion of either the technical questions of immunochemistry or the details of biological interpretation is much too premature and will not be attempted. As an example of some of the problems encountered in such studies, I should like to recount some of the recent experiences in my laboratory and briefly indicate some possible interpretations.

In studies carried out on *Rana temporaria* it was found* by *in vitro* techniques that at least one antigen appears during oögenesis and another between fertilization and hatching. It was found that embryos grown in antisera directed against the supernatant obtained after centrifugation of tail-bud homog

* Flickinger and Nace, 1952; Brachet, personal communication.

TABLE 2
SOME IN VIVO STUDIES OF EMBRYONIC ANTIGENS

Author	Organism tested	Antisera against	Test procedure	Response observed	Specificity and remarks
Oliver Gonzales, 1940	*Trichinella*	Larvae homogenates	Larvae culture	Precipitates in medium Immobilization Disintegration Death	Stage specificity
Perlmann, 1954	Sea urchins	Egg homogenates & fractions Sperm	Egg culture	Jelly coagulation Eggs denuded Activation	Species and tissue spec. Preterminal reversibility
Parks, 1946	Anura	Tadpole saline soly. fraction	Tadpole culture	Culture turbidity Tail pathology Death	Species specificity
Brachet, 1950b	*Rana*	Tadpole granules & supernatant	Embryo culture	Immobilization cytolysis	Preterminal reversibility 'large granules' most effective
Clayton, 1951, 1953	*Triturus*	Developmental stages & embryo regions	Embryo culture Explants	Abnormality vs. death	Stage & tissue specificity
Flickinger & Nace, 1952	*Rana*	Developmental stage fractions	Embryo culture Neur. crest culture Blastomere & blastula inj.	Immobilization cytolysis	Preterminal reversibility Stage & species specificity
Spiegel, 1954	*Rana triturus*	Developmental stage fractions	Reaggregation of dissociated cells	Inhibition of reaggregation	Species and tissue specificity
Kimura, 1927	Chick Duck	"Embryo" pulp	Embryo tissue culture	Growth suppression Cytolysis	Some species specificity
Neter *et al.*, 1935 Witebsky *et al.*, 1935	Chick Duck	Forssman antigen	3 d. embryo *in toto*, *in vitro*	Vascular effects Death	Species specificity
des Ligneris, 1936	Chick other	"Embryo" pulp	Embryo tissue culture	Cytological damage	
Weiss, 1939b, 1947, 1953 Weiss & Wang, 1941	Chick	Adult chicken liver, kidney and muscle	*In ovo* injection various stages	Growth effects	Relative organ spec.
Sigurdsson, 1940, 1942	Chick	"Embryo" pulp	8 & 10 d. embryo heart culture	Growth effects	Growth depression
Burke *et al.*, 1944	Chick	Adult lens	Injected embryos	Cellular, tissue pathology	Specific eye defects

Grunwaldt, 1948, 1949	Chick	Hatched chick brain fractions	Embryo brain culture	Growth inhibition Cellular, tissue pathology	Stage & tissue specificity
Harris, 1948	Chick Mouse Rat	16 d. chick, new born mouse & rat heart grafts	Embryo tissue culture	Cellular pathology	Species & tissue specificity
Ebert, 1949, 1950	Chick	Adult chicken brain, heart, & spleen	Spratt embryo culture	Tissue damage	Tissue specificity
Pomerat, 1949	Chick	Spleen homogenate	*In ovo* injection	Growth effects Abnormalities	"Protection" of circulatory system
Lippman *et al.*, 1950	Chick Rat	Adult rat kidney Glomerular nephritis serum, human	Embryo tissue culture	Loss of function Cellular path. Death	Tissue, but not species, specificity
Nettleship, 1953	Chick	Whole embryo brei	*In ovo* injection	Growth suppression Malformations Death	Stage & tissue specificity
Niven, 1929	Mouse, rat Chicken G. pig	17 d. mouse embryo	Embryo tissue culture	Cellular pathology	Not organ specific Specific to mouse and rat
Narita, 1935	Rat	Rat sarcoma	Fetal heart culture	Death	Tumor specific
Pomerat, *et al.* 1945	Rat G. pig Chick	Spleen homogenate	Embryo spleen culture	Inhibition	

enates at 18,000 × gravity were specifically immobilized and cytolyzed along anteroposterior, dorsoventral gradients, when they reached the tail-bud stage.

Before continuing the studies in this country, it was necessary to confirm these observations on *Rana pipiens*. Two problems were encountered: first, the same preparation and injection techniques which produced usable antisera in the previous study proved to be strong anaphylactogens and killed the local rabbits; and, second, the normal sera from these rabbits were more "toxic" to the embryo than were those encountered previously. These problems were solved in time to use the antisera on the last embryos of the 1954 season by utilizing Freund's (1951) adjuvant, subscapular single injection procedures, and by ammonium sulfate precipitation and dialysis of the rabbit serum to obtain the antibody containing fractions and control the ionic concentration of the medium.

In general, the results of *in vivo* cultivation of *Rana pipiens* in antisera to antitail-bud supernatant sera were similar to those obtained with *Rana temporaria*, although the number of tests which have been made are insufficient to be certain of their identity. Further *in vitro* tests, application of absorption procedures, and specificity determinations are necessary. However, abnormalities of many kinds, particularly involving the cephalic region, have been observed, and longer exposure or utilization of higher concentrations of antibody led to "cytolysis," disintegration, and death, conditions that were not found in the normal medium or in "normal rabbit serum" controls.

Particularly interesting is the observation that the initial phases of this "specific cytolysis" resemble the shedding of pigment-free mesectoderm cells described by Holtfreter (1943), when embryos of various amphibian species are cultured under adverse conditions which lead to a disruption of the surface coat of the embryo. It seems possible that we might be dealing here with antigens contained in this coat which, Holtfreter (1949) has indicated, plays such an important function in the morphogenetic movements of gastrulation and neurulation, as well as in providing an osmotic barrier for the embryo. Holtfreter has suggested that this coat consists mainly of proteins combined with calcium, and it would be interesting if the observations reported here should prove to indicate a change in the antigenic specificity of these proteins, which might be correlated with the completion of the morphogenetic movements that have occurred just prior to the development of the tail-bud. Doctor Grobstein (1955a, b) has focussed attention on such matrix changes in development, and Doctor Spiegel, using *in vivo* serological techniques, has presented evidence on the role of surface antigens in cell adhesion in sponges (1954a) and in amphibians (1954b), some of which he has reviewed in this monograph (1955c).

These are a few of the observations the significance of which will be known only after further data are available from the gamut of available techniques.

With the hope of exciting results, waiting only for those who will do the work, perhaps it will be possible to repudiate the sense, if not the truth, of the published remarks of a scientist who states: "in searching for the active antigen, two procedures are available. . . . This procedure (one of them) has many drawbacks. For one thing, it requires a rather large amount of material that often is difficult to obtain. Several animals have to be immunized each time,

as the animals differ greatly in their reaction to the injected substance. Further, this procedure takes a long time."

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THE REAGGREGATION OF DISSOCIATED SPONGE CELLS

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Introduction

Within the past 50 years, specific surface antigens have been demonstrated in many types of cells: for example, bacteria, protozoa, gametes of echinoderms, amphibia and mammals, and erythrocytes of mammals, fish, and birds. But it is still uncertain, except for gametes, what use these components of the cell surface are to the organisms in which they occur. With respect to erythrocyte antigens, Boyd (p. 188, 1947) says "... absolutely nothing is known of any normal physiological function of the group substances ...."

It has been suggested by Tyler (1940, 1942, 1946, 1947) and Weiss (1941, 1947, 1950) that, in multicellular forms, cell surface antigens may perhaps be an important factor in the cohesion of cells within embryonic and adult tissues, and that changes in these antigens might conceivably bring about the changing cohesiveness important in the cell and tissue movements of morphogenesis and in the sustained output of isolated cells: *e.g.*, lymphocytes, from the germinal tissues of adults. The hypothesis, in somewhat vague form, goes back at least to Loeb (1922).

There is one surprising feature in this concept of the role of surface antigens in metazoa: the evidence cited in support of it comes mainly from experiments on tissues in which surface antigens had not been demonstrated. Thus Tyler (1947) cites mainly the selective reaggregation of experimentally dissociated sponge cells and the segregative movements in experimental combinations of embryonic amphibian tissues (Holtfreter, 1939). Recently (Spiegel, 1954a, 1954b), we have been able to demonstrate the presence of specific surface antigens in these tissues.

The remarks which follow are concerned primarily with investigations on the role of surface antigens in the reaggregation of dissociated cells of two marine sponges: *Microciona prolifera* and *Cliona celata*.

Normal Reaggregation and Segregation of Sponge Cells

Wilson (1907) first demonstrated that a sponge could be dissociated by pressing it through bolting cloth into isolated cells which would form a number of reaggregates within a day's time. Galtsoff (1923, 1925, 1926, 1929) studied the effects of ions, pH, temperature, *etc.* on the reaggregation process and made the first attempt to express the process quantitatively. Other aspects of the phenomenon have been studied by Wilson (1910, 1932), de Laubenfels (1927, 1932), and Brøndsted (1936), among others.

When observed as soon as possible after disaggregation into sea water, many of the cells are found to be on the bottom of the container the others slowly settle down. On the basis of movement, the cells may be divided into two classes: (1) the amebocytes—those cells that move by putting forth pseudo-

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podia that are hyaline and devoid of any granular content; and (2) the choanocytes or collar cells — cells which move about by means of flagella. This division may not be as sharp as has been previously thought, since we have noted that, in two species of West Coast sponges, *Hymeniacidon sinapium* and *Tetilla mutabilis*, the collar cells, in addition to possessing a beating flagellum, are capable of ameboid movement (Spiegel and Tyler, unpublished).

The cells begin to move almost immediately, the amebocytes moving at about 0.6 to 3.5 μ minute and changing direction, apparently at random, roughly three times per hour (Galtsoff, 1925). The cells generally coalesce upon coming in contact with one another. The cell surfaces of the apposed cells join together zipperwise, proceeding from the area of first contact. After 5 to 10 minutes, the surface of union between any two cells is indistinct, and evidence (Spiegel, 1954a) has been presented which indicates that the outer hyaloplasm forms a common matrix for the inner granuloplasms, which remain separate and distinct from one another.

Larger and larger aggregates are formed as new contacts occur (FIGURE 1). The number and size are dependent on the density of the original suspension,

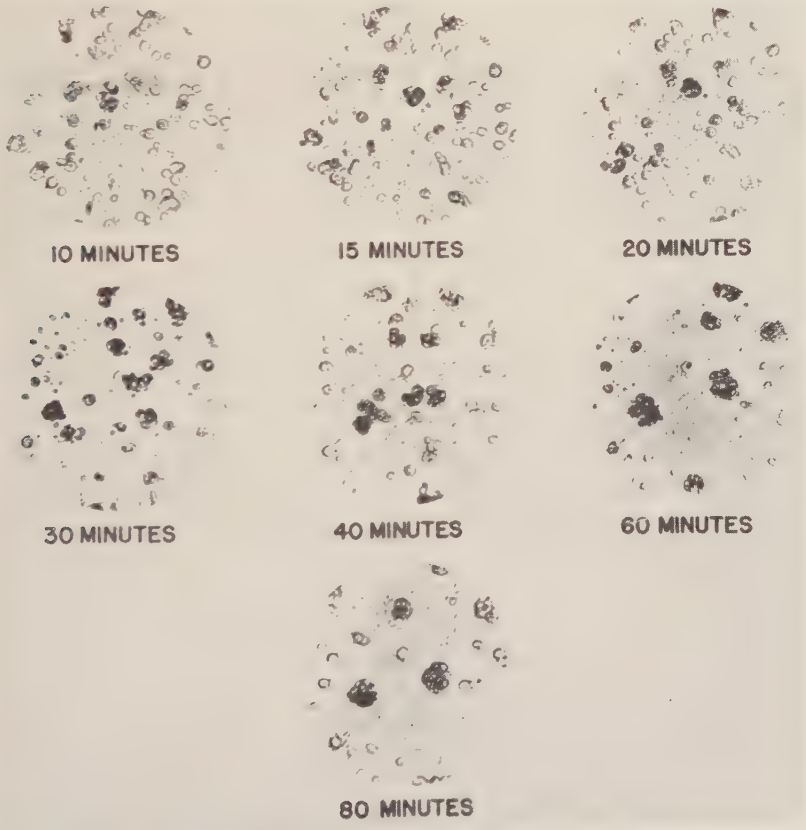


FIGURE 1. Normal reaggregation of dissociated *Microciona* cells in sea water. ×120.

temperature, osmotic pressure, ionic composition of the medium, pH, *etc.* (Galtsoff, 1923). Two processes can be distinguished: intake of single cells by aggregates, and coalescence of aggregate with aggregate.

If one makes a mixed suspension of two species of cells, *e.g. Microciona* and *Cliona*, it can readily be seen that, 24 hours after dispersal, the aggregates formed consist of only the one species or the other (Wilson, 1910; Galtsoff, 1925; de Laubenfels, 1927). It should be pointed out, however, that this segregation is preceded by a temporary intermixture of cells of the two species, followed by a later sorting-out. After sorting-out has taken place, the aggregates, readily distinguished by the red color of *Microciona* and the yellow color of *Cliona*, are *never* a homogeneous mixture of cells of both species. It should be admitted that a few cells of one type in an aggregate prevailingly of the other species could not be distinguished, and it is conceivable that such mixtures occur.

The *Microciona* aggregates formed in a mixed suspension are more numerous and correspondingly smaller than in a pure *Microciona* suspension. The same holds true for the *Cliona* aggregates. One can occasionally observe an aggregate of *Microciona* and one of *Cliona* in close contact, but their hyaloplasms remain separate, and the two fail to adhere.

There are at least two factors which would lead to a smaller size of aggregates in mixed suspensions: (1) contacts which would lead to a permanent increase of aggregate size in a pure suspension are cancelled out in a mixed preparation by the later sorting-out where interspecific contacts had occurred; and (2) to some extent, each species may also interfere mechanically with the other's aggregation, as Galtsoff (1925) has shown glass particles and starch grains to do.

Effect of Calcium on Reaggregation

The importance of calcium as an agent for holding cells together was first pointed out by Ringer in 1890. He observed, in tadpoles and in algae, that distilled water loosened the substance binding the cells together, and that this loosening could be counteracted by bicarbonate of lime. Herbst (1900) observed that sea-urchin blastomeres would not adhere to each other in calcium-free media. Lilly (1906) studied the action of calcium ions on the ciliated epithelium of *Mytilus edulis*, and Chambers (1940) on the epithelium of the chicken embryo. Galtsoff, in 1925, made an extensive study on the effect of ions on the reaggregation of dissociated sponge cells and reached the conclusion that calcium was necessary for normal reaggregation. He was able to obtain stable cell suspensions in calcium-free media. De Laubenfels (1932) reported that reaggregation in the sponge *Iotrochota birotulata* is impeded in media outside a limited range of calcium concentrations. In contrast to these previous reports, Agrell (1951) was unable to maintain *Halichondria panicea* suspensions as isolated cells in citrate or oxalate solutions.

In view of this inconsistency, it seemed desirable to repeat some of the previous work on the function of calcium. Three types of experiments were carried out, using the sponge *Microciona*: in calcium-free media; in the presence of a calcium-complexing agent, ethylene diamine tetra acetic acid; and in high

calcium solutions. Experimental details are reported elsewhere (Spiegel, 1954a). The results were as follows: in calcium-free media and in the presence of a calcium-complexing agent, little or no reaggregation was noted. The cells do not move and no coalescence occurs. If, however, the vessel containing the cell suspensions are swirled, large macroscopic aggregates were formed. It was concluded, therefore, that the failure of reaggregation to take place was not due to a lack of adhesiveness but to the loss of movement. Galtsoff (1925) also reported that reaggregation is inhibited by an excess of calcium in the medium. In experiments under identical conditions with those of Galtsoff's, *i.e.*, increasing the calcium of the medium to 4 1 times that of sea water, we found no effect of excess calcium on the reaggregation.

If calcium played any major part in the adhesion which is a necessary preliminary to coalescence, one would expect it to be reflected by some changes in reaggregation with changing calcium in the medium, especially where calcium is presumably removed both from medium and cells by the complexing agent. An essential role of calcium that could not be detected in this way is conceivable but not likely. Hence, one must conclude that, in the selective adhesiveness of sponge cells, calcium is much less important than previously thought, and perhaps not normally involved at all.

Effects of Antisera on Reaggregation and Segregation

Ebert (1952) has pointed out that the failure of immunological analyses to reveal specific differences in the organ antigens of the early embryo has led to the adoption of a second approach to the problem of determining the time of appearance of tissue-specific antigens. This approach involves the treatment of the early embryo either by injection of, or culture in, specific antiorgan sera, in the hope that it might prove to be a more sensitive method than the usual immunological techniques employed for detecting specific antigens. It has been used by Burke *et al.* (1944) and Ebert (1950, 1951, 1952, 1953) to study the development of specific organ antigens in the chick, and by Flickinger and Nace (1952) and by Clayton (1953) in amphibian embryos. To our knowledge, however, this approach has not been used extensively to test a corollary of the Tyler-Weiss hypothesis, namely that contiguous cell surfaces are held together by antigen-antibodylike forces.

Results which are in support of the Tyler-Weiss hypothesis have recently been reported by Spiegel (1954a), who studied the effects of specific antisera on the reaggregation of the cells of two marine sponges: *Microciona prolifera*, the red encrusting sponge, and *Cliona celata*, the yellow sulfur sponge.

Three classes of antisera were produced in rabbits (for details see Spiegel, 1954a). One group of animals received injections of a suspension of *Microciona* cells; a second, of *Cliona* cells; and a third group received injections of a mixed suspension of cells consisting of *Microciona* and *Cliona* cells; a fourth, uninjected group, served as controls. The antisera will henceforth be designated as anti-*Microciona*, anti-*Cliona*, anti-*Microciona* : *Cliona*, and normal serum, respectively.

Reaggregation was followed at ×430 magnification by counting, at intervals, the number of aggregates in each of five microscope fields taken at random,

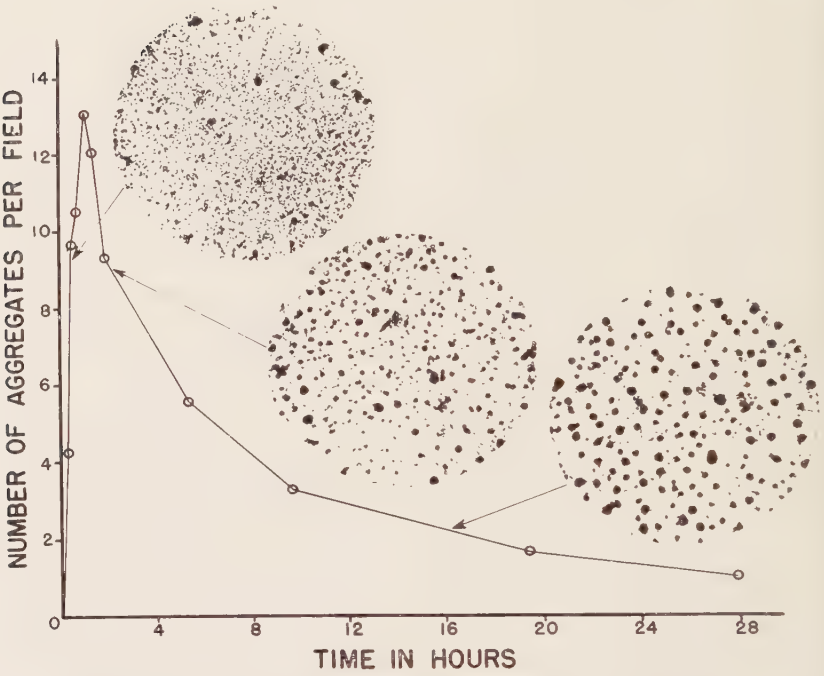


FIGURE 2. Reaggregation curve of dissociated *Microciona* cells in normal serum.

and the values averaged. An aggregate was defined as any coalesced group of four or more cells. Typical results over a 28-hour period, in normal serum, are shown in FIGURE 2. Such a figure is called a reaggregation curve, and each of its ordinates, an aggregate count. The curves are reproducible under standard conditions and show an increase in number of aggregates (of small size) to a maximum at about one hour, followed by a decrease in number (fusion of aggregates with corresponding increase in size).

The results with antiserum are as follows:

Microciona in anti-Microciona serum. Reaggregation curves for *Microciona* cells during the first three hours in normal serum, and two in anti-*Microciona* sera are shown in FIGURE 3 with photomicrographs at 2.75 hours. The curve in normal serum is as described. In anti-*Microciona* serum, the number of aggregates increases with time reaching peak values of 26 and 27 aggregates per field at 2.25 hours, as compared with a value of 13 in normal serum, and then levels off. After this period, the number of aggregates, in antiserum, does not change significantly.

In normal serum at 2.75 hours, less than 10 per cent of the cells remain dissociated. The rest of the cells are included in aggregates of roughly 100 to 200 cells. In antiserum, about 90 per cent of the cells are still dissociated at that time, and most of the aggregates contained fewer than 20 cells; a few contained 100 or more cells.

In antiserum, even the largest aggregates had irregular shapes and borders,

whereas in normal serum the aggregates are circular, oval, or, where two are fusing, dumbbell-shaped. A second difference was that, in antiserum, a large proportion of cells were in loose clusters. Adjacent cells were scarcely more than tangent, with very little surface in contact.

It is evident from these observations that much less reaggregation occurs in antiserum than in normal serum, even before two hours. Spiegel (1954a) has shown that the striking increase in aggregate counts during the first two hours in antiserum can be attributed to settling of aggregates present at the start before antiserum has been added. There is little reason to conclude that any reaggregation of *Microciona* cells occurs in anti-*Microciona* serum at any time. In fact, estimates of the number of free or dissociated cells indicate that the aggregates present from the start tend to shed cells.

This failure of reaggregation, and the possible further disaggregation, were not due to death of the cells. The cells in antiserum exhibit normal pseudopodial movement. In fact, cells which have been in antiserum for periods

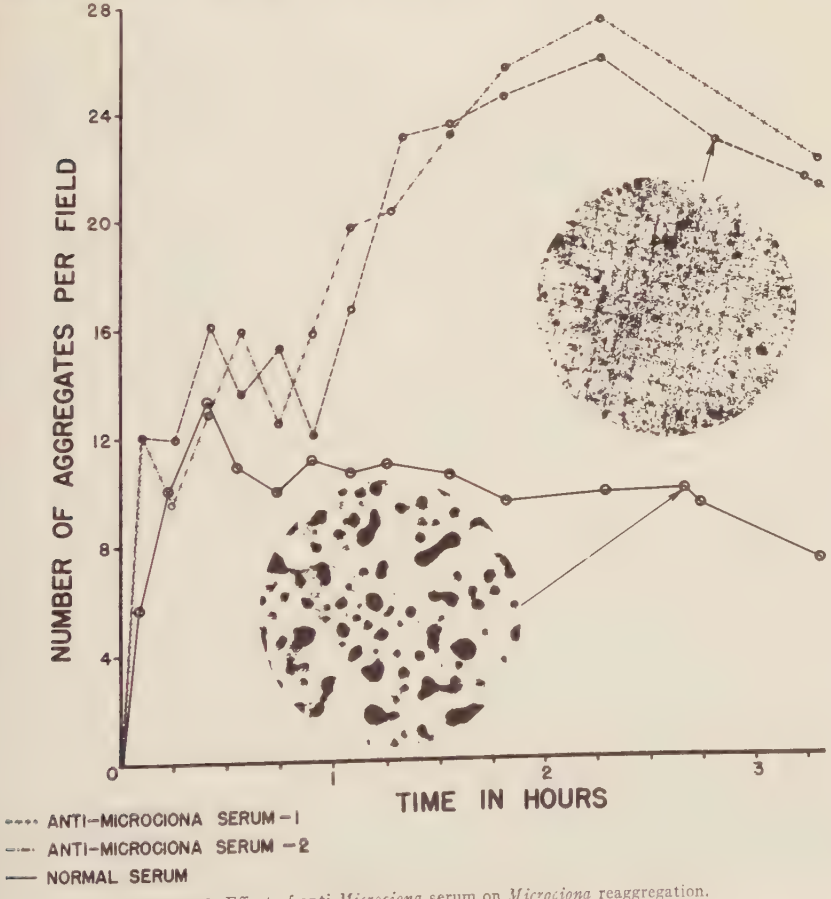


FIGURE 3. Effect of anti-*Microciona* serum on *Microciona* reaggregation.

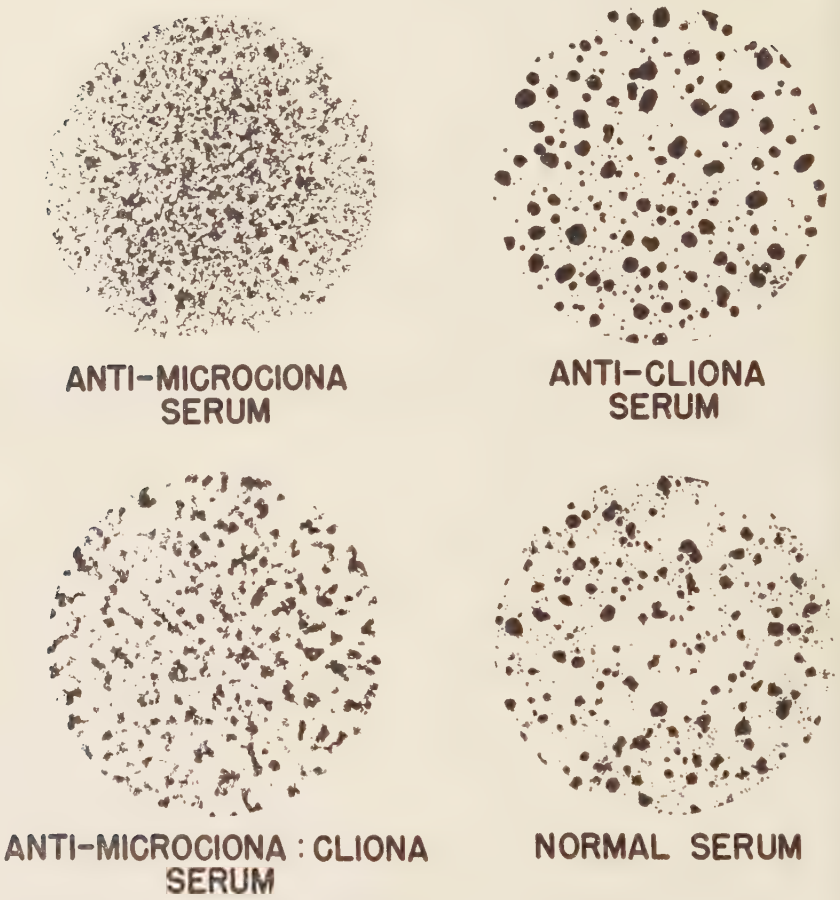


FIGURE 4. Effect of antisera on *Microciona* reaggregation. X12.

ranging up to 12 hours can be washed once, transferred to normal serum, and will reaggregate normally.

Other Combinations of Sponge and Antiserum

Microciona (FIGURE 4). In anti-*Cliona* serum there were no signs that reaggregation had been inhibited. The aggregates in anti-*Cliona* serum were as large or larger than normal.

In anti-*Microciona* : *Cliona* serum, *Microciona* formed aggregates of number and size intermediate between those in normal serum and in anti-*Microciona* serum; reaggregation was partly inhibited.

Cliona (FIGURE 5). There were more, and smaller, aggregates in anti-*Cliona* serum than in normal serum, indicating an inhibition of reaggregation analogous to that of *Microciona* in anti-*Microciona* serum.

In anti-*Microciona* : *Cliona* serum, there were fewer aggregates than in anti-

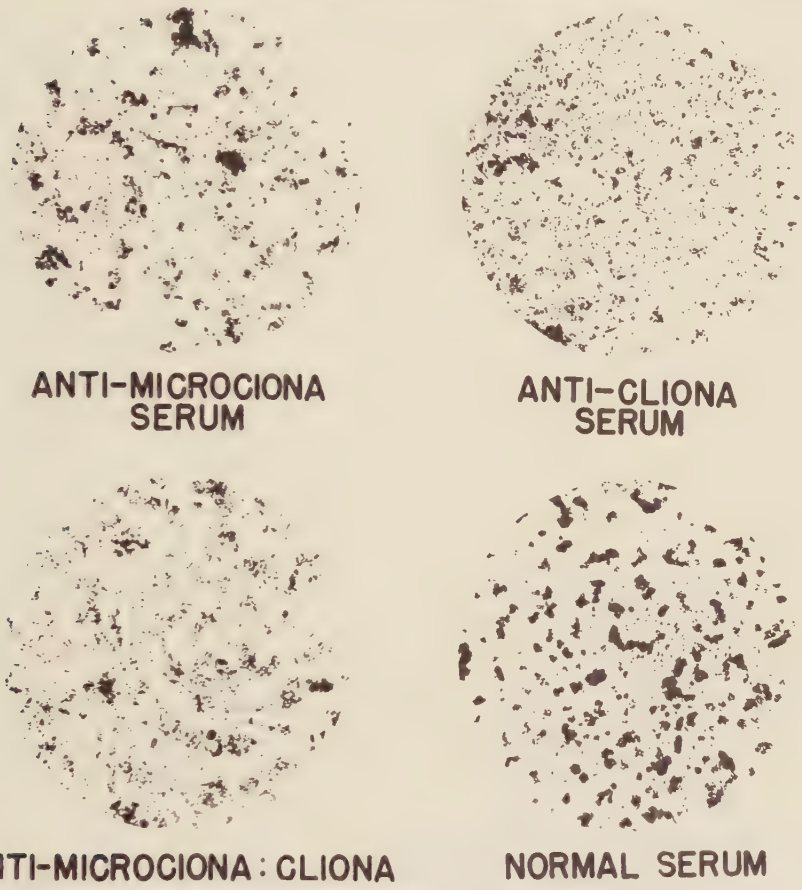


FIGURE 5. Effect of antisera on *Cliona* reaggregation. ×12.

Cliona serum but more, and smaller, than in normal serum. The inhibition was partial, like that of *Microciona* in the same serum.

With anti-*Microciona* serum there were fewer and larger aggregates than in anti-*Cliona* serum, but smaller than in normal serum, and scarcely larger than in anti-*Microciona* : *Cliona* serum.

Microciona-Cliona mixtures (FIGURE 6). In normal serum, the initially mixed cells sorted out. The *Microciona* aggregates were smaller than are formed by pure *Microciona*, as were also the *Cliona* aggregates. The probable reason for this smaller size has already been suggested above and has nothing to do with their surface antigens.

In anti-*Cliona*, as in normal serum, there were no mixed aggregates, and the *Microciona* aggregates were normal, though smaller than those formed by *Microciona* alone in normal or in anti-*Cliona* serum. The *Cliona* components of the suspension were much like *Cliona* in anti-*Cliona* The results are what

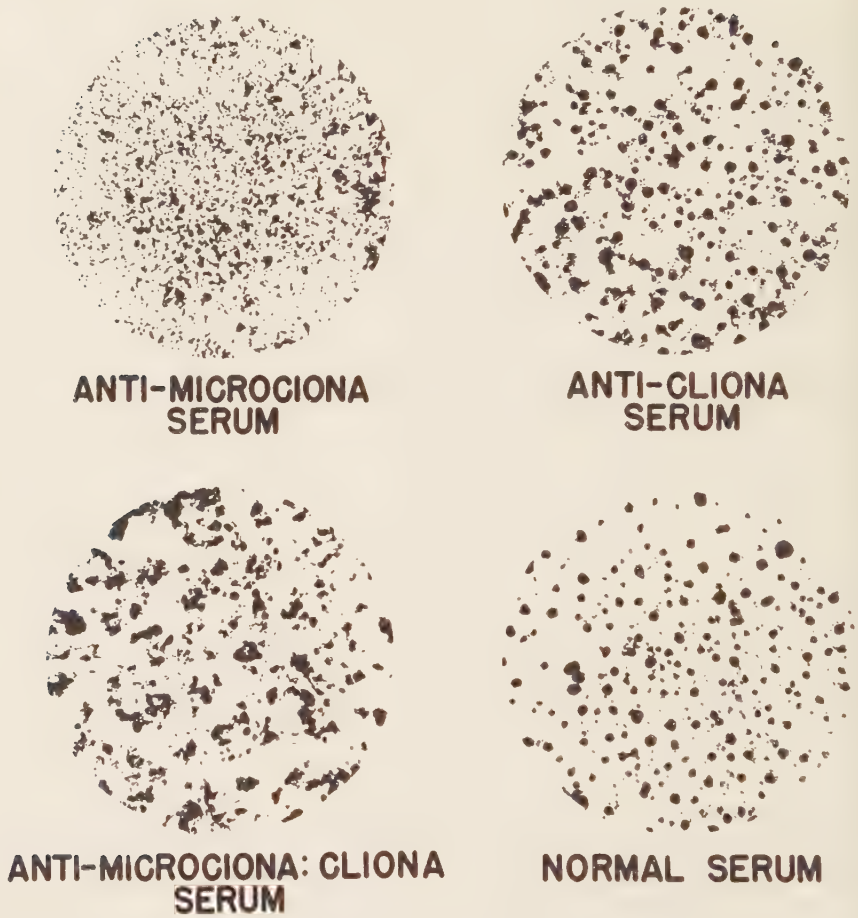


FIGURE 6. Effect of antisera on the reaggregation of mixtures of *Microciona* and *Cliona* cells. ×12.

would be expected if anti-*Cliona* serum contained antibodies to surface antigens of *Cliona* but not *Microciana*.

In anti-*Microciona* serum, the mixed suspension did not form any large aggregates of the *Microciona* type, but many isolated cells and small clusters were seen. The larger aggregates were found to consist of *Cliona* cells. Most of them were about as large as those formed by *Cliona* alone in anti-*Microciona* serum. They were smaller, though, than those of *Cliona* alone in normal serum. This difference can be interpreted as a consequence of the cancelling of previous associations by ejections of *Microciona* cells from mixed clusters as suggested above for mixed cells in normal serum.

In anti-*Microciona* : *Cliona* serum, a result was obtained diametrically opposite to the effect of the other antisera on pure or mixed suspensions. There were large aggregates of general shape intermediate between *Microciona* in normal serum and *Cliona* in normal serum. Many of the aggregates were

much larger than in any other cell-serum combination. At the time of the experiment, it was noted that: (1) cells, or cell groups, of the two species were intermingled apparently at random throughout the aggregates. At least, there were no patches of red *Microciona* cells or of colorless *Cliona* cells, but the aggregates were of a uniform, intermediate color; (2) the aggregates had a somewhat porous structure; in the reticulum between pores, the cell-to-cell contacts seemed to be about as close as in either *Cliona* or *Microciona* in normal serum.

The effect of the anti-*Microciona* : *Cliona* serum could not be reproduced by mixing anti-*Microciona* serum with anti-*Cliona* serum (FIGURE 7). In the mixture of antisera, both cell-species are partially inhibited, as would be expected from the results obtained with either serum alone.

The results show that reaggregation is inhibited by the presence of homologous antibodies in contrast to the usual phenomenon of agglutination. There are several conditions under which agglutination may not occur, even though specific reaction takes place between surface antigens and homologous antibodies.

(1) *Intercellular cement.* Under this condition, the antibodies combine

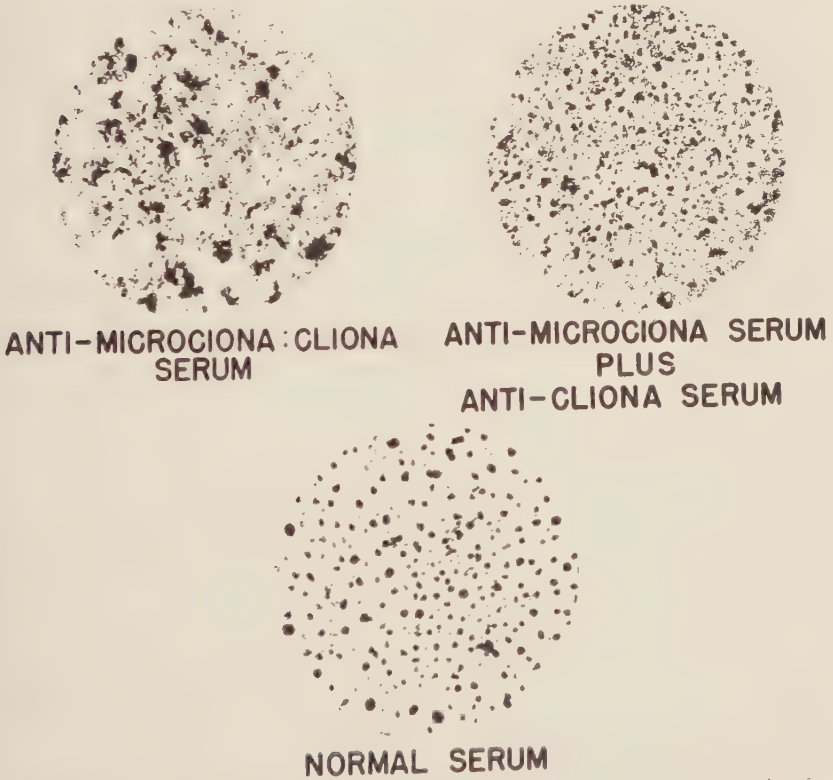


FIGURE 7. Effect of a mixture of anti-*Microciona* serum plus anti-*Cliona* serum on the reaggregation of mixtures of *Microciona* and *Cliona* cells. ×12.

with an intercellular cement, and either precipitate or inactivate this substance, rendering it inactive with respect to its use as a binding agent of cells. De Laubenfels (1932) has reported this intercellular cement or "slime" to be present in the sponge *Iotrochota birotulata*. In investigations on seven species of marine sponges, we have never observed the presence of this "slime" or intercellular cement, and no precipitate has been noted upon the addition of antiserum. Furthermore, the inactivation of this cement would not account for the apparent agglutination of mixed suspensions of cells by the homologous antiserum.

(2) *Extreme antibody excess.* One could assume that the sera used were of such high titer that the vast majority of the cells are completely saturated with agglutinin before cell-to-cell contacts are made. Since all of their receptor sites are now occupied, the cells are incapable of reacting with each other. It does not seem likely, however, that all of the antisera used, with the exception of those produced versus mixed cell suspensions, would have been of this order of magnitude of antibody concentration.

(3) *Univalent antibody.* Under this condition, it is assumed that the antibodies formed by a rabbit in response to injections of sponge antigen are predominantly of the univalent type. This type seems to be found in Rh antisera (Weiner, 1944) and other immune sera (see references in Tyler, 1945). Although these antibodies could combine with surface antigens, agglutination would not take place, since each antibody molecule possesses only a single reactive group. As in condition (1), this hypothesis would be adequate for explaining the inhibition of reaggregation by homologous antiserum, but offers no explanation for the apparent agglutination of mixed suspensions of cells by the homologous antiserum.

(4) *Structure of cell surface.* The surface of a sponge cell is observed to change its shape, as evidenced by pseudopodial formation, and it seems reasonable to assume that this flexibility and movement is sufficient to allow the valence groups of a multivalent antibody molecule to contact two or more of the receptor sites of the *same* cell surface. Very few, if any, valence groups would then be available for reaction with receptor sites on other cells. This phenomenon can account for the absence of agglutination and the inhibition of reaggregation.

It can also account for the clumping obtained with mixed cells (of the two species of sponge) in an antiserum versus mixed cells. Here, it is assumed that heteroligating antibody molecules are formed. If, for simplicity, we consider only two valences of an antibody molecule, then an antibody molecule produced in response to injections of *Microciona* cells would have two anti-*Microciona* valences. Similarly an antibody molecule produced in response to *Cliona* would have two anti-*Cliona* valences. In an antiserum versus a mixed cell suspension, a certain proportion of antibody molecules would have one valence directed towards *Microciona* cells, and the second valence towards *Cliona* cells. These molecules are called heteroligating and could react at one end with cells of one species of a mixed suspension, leaving a valence group free to contact cells of the other species, thereby forming mixed aggregates.

The failure of ostensibly multivalent antibodies to cause agglutination has

been noted in several immunological systems. Gleeson-White *et al.* (1950) and Coombs *et al.* (1951) have studied the reactions of ox red cells in rabbit or guinea-pig antiox sera. The red cells of most oxen fail to agglutinate in such sera. Coombs *et al.* suggest that the failure is due to a deep location of the antigens, so that a single antibody molecule, even if multivalent, after combining with one cell, cannot reach receptors of a second cell.

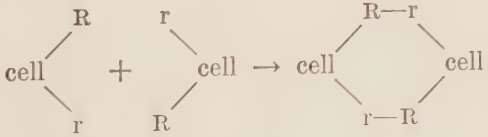
Their hypothesis does not, however, enable us to account for the agglutination of mixed cells by antimixed cells serum. It was therefore modified in terms of a single antibody molecule reacting with different parts of the same cell surface. Apart from the validity of the interpretation, it appears clear from the work of Coombs *et al.* that there can be failure of agglutination, even in the presence of multivalent antibody, with certain types of cells.

The results obtained from the experiments with antisera offer strong support for the Tyler-Weiss hypothesis that specific surface antigens play an important role in the selective adhesion of cells. There seem to be only two possible ways of operation of these surface antigens in adhesion:

(1) Through an extrinsic binder like calcium, *e.g.* cell-surface antigen-Ca-surface antigen-cell, or some intercellular cement, *e.g.* cell-surface antigen-cement-surface antigen-cell. The failure of calcium complexing agents to prevent reaggregation and the absence of any "slime" or cement in the forms studied makes this hypothesis unlikely.

(2) By some type of direct bonding between two intramolecular configurations with the reciprocal structural relationship of antigen and antibody. These reciprocals can exist either within a single molecular species or in distinct substances. Since cells of the same type readily adhere to each other, we can assume that a single cell has *both* reactants on its surface. Designating the two reactants as *R* and *r*, adhesion implies that *R* and *r* are so arranged on the cell surface that, under suitable conditions, they can combine with *r* and *R* of another cell. Within the surface of any one cell, *R* and *r* must be so held in position that they cannot combine with each other.

The structure of a cell surface, then, with respect to adhesion, would be crudely analogous to a balloon on which many snap-fastener parts have been attached, male and female separately but interspersed, and the unit reaction in adhesion would be



The *R*—*r* bonding would be that of antigen to antibody; the cell $\begin{matrix} R \\ \diagup \\ \diagdown \\ r \end{matrix}$ bonding would be whatever holds the components of a cell surface in position.

Autoantibodies to Surface Antigens

It has also been suggested by Tyler (1947) that reciprocal surface configurations, in addition to explaining the specific adhesion or nonadhesion of cells and tissues and their organization into organ systems, may also be involved in cell differentiation. It is known, from the studies of blood cells, that the surface antigens are gene-determined. Tyler points out that, since all of the somatic cells of an individual are supposed to contain the full complement of genes, one should expect to find the same surface antigens on all cells.

From the blood-group work, however, we know that, although some antigens, *i.e.* human erythrocyte antigens *A* and *B*, are found in almost all cells of the body, other antigens, such as the *M* and *N* blood groups, are restricted to the blood cells. Tyler accounts for this segregation of surface antigens in the following manner. Although all of the genes may be active in producing antigens, they may do so at different rates and at different times, and therefore certain antigens do not reach the cell surface. Differentiation may involve differences in the rates of production of different antigens; at first, in different regions of the uncleaved egg; later in different blastomeres and embryonic tissues. Production of these substances may occur in an antigen-antibody manner, that is, the large molecular constituents synthesized in a cell would be complementary to the substances comprising the sites of synthesis.

We may, then, expect to find a particular surface substance being represented by the complementary substance on another type of cell or within the same cell. Tyler (1940) has shown this finding to be true in the case of the eggs and sperm of sea urchins and other animals. Here the gelatinous coat surrounding the egg contains a substance called fertilizin, and the surface of the sperm is covered with a layer of complementary substance called antifertilizin. The reaction between fertilizin and antifertilizin is necessary for fertilization to take place (excluding parthogenesis), and it offers an explanation for the specificity involved. Tyler has also demonstrated that an antifertilizin is located in the interior of the sea-urchin egg. It is probable that the egg antifertilizin and sperm antifertilizin are not the same chemically, but are only similar in their ability to react with fertilizin. The autoantivenin present in the Gila monster is an example of an autoantibody in another organism (Tyler, 1946).

If the hypothesis which we have presented to account for the selective adhesion of sponge cells is correct, it appears likely that one should be able to find an autoantibody in sponge cells. The following experiments (in collaboration with Doctor Albert Tyler) were therefore performed.

Two methods of extraction were utilized. The first attempt to extract sponge autoantibodies involved freezing and thawing *Microciona* cells in sea water. This method has been described by Tyler (1940) for the extraction of egg antifertilizin. The extract was centrifuged to remove cell debris, the pH adjusted to 7.8, and the extract tested for its effect on the reaggregation of *Microciona* and *Cliona* cells. The results are as follows:

The extract had no observable effect on the reaggregation of *Microciona* cells. It did, however, agglutinate *Cliona* cells, which formed large macro-

scopic aggregates of irregular shape. Later these aggregates adhered to the substratum, rounded up, and appeared to be developing normally. The active substance is nondialyzable and precipitated out at pH 2.8. It is inactivated by boiling for 15 minutes, and is probably identical with the heteroagglutinins described by Galtsoff (1929).

Spiegel and Tyler (unpublished) have noted that the antifertilizin of sea-urchin eggs is heat stable and is more easily extracted by boiling eggs in sea water than by the freezing and thawing method. It was thought that perhaps this method might be useful for extracting sponge autoantibodies. Extracts were made by boiling *Microciona* cells in sea water for 15 minutes, centrifuging to remove the cell debris and coagulated protein, the pH being adjusted to 7.8.

The method proved to be successful and, when tested on *Microciona* cells, agglutination was observed. The large macroscopic aggregates which formed soon adhered to the glass, rounded up, and developed normally with one striking exception. Approximately four hours after addition of the extract, a precipitate begins to appear around each aggregate which intensifies with time and, about 12 hours after addition, each aggregate seems to be surrounded by a halo. It has not been determined whether this precipitate is extracellular or is within the hyaloplasm.

The extract is nondialyzable, heat stable, and precipitated out of solution at pH 3.0 or by 50 per cent saturated ammonium sulfate. It has no effect on *Cliona* reaggregation. No completely satisfactory explanation can be offered, at present, to account for the agglutination of cells by the cell extract in contrast to the inhibition in rabbit antiserum. Perhaps the arrangement of the active sites on the autoantibody is such that they cannot combine with antigens of the same cell.

The Production of Heteroligating Antibody in Rabbits

One conclusion reached as a result of the experiments with rabbit antisera is of considerable immunological interest: when *Microciona* and *Cliona* cells are injected together into a rabbit, some antibody molecules are produced with one or more groups reactive to *Microciana* antigens and one or more groups reactive with *Cliona* antigens.

Perhaps the most favorably regarded theory of the mechanism of antibody formation is the one assuming that antibody is synthesized in spatial contact with antigen, resulting in a molecule bearing a reciprocal configuration to that of antigen (Breinl and Haurowitz, 1930; Alexander, 1931; Mudd, 1932). A more recent modification of this theory (Pauling, 1940) states that the number, kinds, and sequences of amino acids making up the antibody molecule are identical with normal globulin. It is only the way in which the two ends of the polypeptide chain are folded to form the globular molecule which makes for antibody specificity. Both ends of the chain are always influenced by the same antigen molecule and never by two different molecules.

In attempting to furnish experimental support for this theory, it is of interest to learn if antibody molecules are formed having separate reactive sites for two or more antigen molecules (heteroligating antibody). The earlier evi-

TABLE 1

NUMBER AND TYPE OF AGGREGATES FORMED BY MIXTURES OF CHICK AND SHEEP ERYTHROCYTES IN VARIOUS SERA

Experiment number	Serum*	Number of chick aggregates per cu. mm.	Number of sheep aggregates per cu. mm.	Number of mixed aggregates per cu. mm.	% Mixed aggregates
4	AC	480	6	0	0.0
	AS	0	980	0	0.0
	ACS	535	382	130	12.4
	NS	0	0	0	0.0
	AC + AS	209	464	3	0.4
5	AC	290	10	0	0.0
	AS	0	594	0	0.0
	ACS	483	136	85	12.1
	NS	0	0	0	0.0
	AC + AS	202	406	0	0.0

* AC = antichick serum; AS = antisheep serum; ACS = antichick:sheep serum.
NS = normal serum; AC + AS = antichick plus antisheep serum.

dence (Dean, Taylor, and Adair, 1935; Haurowitz and Schwerin, 1943) seems to indicate that heteroligating antibody is not formed. More recent work, however, with Rh antisera by Race *et al.* (1948), with bacteriophage by Roesel (1951), and with erythrocytes by Dodd (1952) indicates that such antibodies are formed in response to the simultaneous injection of two or more antigens.

In view of the contradictory results, a further investigation of the problem seemed warranted, using agglutinating systems which have been extensively investigated. The following experiments, performed in collaboration with Doctor Donald R. Charles, were carried out using antisera produced in rabbits versus sheep erythrocytes, versus chick erythrocytes, and versus a mixture of sheep and chick cells.

Methods comparable to those of the sponge experiments were used. Mixtures of chick and sheep erythrocytes were added to the various sera, and counts were made of the number of clumps of cells composed of (1) chick cells, (2) sheep cells, and (3) mixed clumps (clumps consisting of both chick and sheep cells). Results of two experiments using sera diluted 1:25 with saline are shown in TABLE 1.

At dilution of 1:25, antichick and antisheep sera are specific, and cross agglutination does not occur to any appreciable extent. It is apparent that only in the serum versus both cell types were mixed aggregates formed in significant number. In mixtures of antichick and antisheep sera, both cell types were agglutinated, but aggregates composed of both chick and sheep cells were only 0.4 per cent of the total in experiment 4, and 0 per cent in experiment 5. In antichick-sheep serum however, 12.4 per cent of the aggregates formed were mixed in experiment 4 and, in experiment 5, 12.1 per cent of the aggregates were mixed.

Thus, the results with chick and sheep cells, as in the case of the sponge experiments, indicate that heteroligating antibodies *are* formed in rabbits in response to the simultaneous injection of two or more antigens.

The Effects of Antisera on Reaggregation of Embryonic Amphibian Cells

It should be emphasized that, in the sponge experiments, we have been dealing with the cell recognition and discrimination that occurs between cells of different species. But this recognition and discrimination also exists in the cells within a species. This latter type of discrimination has been reported to occur in the reindividuation of a sponge aggregate by Galtsoff (1925) and by Wilson and Penney (1930) and, in the case of embryonic amphibian cells, by Holtfreter (1939). We have not, as yet, been able to obtain pure suspensions of the various cell types in sponges, and so further investigations along this line were carried out using embryonic amphibian cells.

Holtfreter (1939) has demonstrated that small pieces of ventral ectoderm and endoderm, isolated together with a little mesoderm, form an aggregate which holds together, with endoderm forming part of the surface and ectoderm the remainder. If more ectoderm is provided, sufficient to enclose the mesoderm and endoderm, the endoderm will form a tubular structure within the mesoderm.

Portions of endoderm and ectoderm isolated together, without mesoderm, at first cling together but later tend to separate. This self-isolation begins after two days in *Rana pipiens* and, after 3 to 4 days, in *Ambystoma punctatum*. Complete isolation occurs after four days in *Rana pipiens* and, after 6 to 9 days, in *Ambystoma punctatum*. The mesoderm behaves therefore as a "connective tissue."

These tissue affinities are apparently not species-specific at early stages of development, and may be seen in practically any xenoplastic combination. For example, Townes (unpublished) has found that the ectoderm of *Rana pipiens* gastrulae with the endoderm of *Ambystoma punctatum* or *Triturus torosus* will exhibit self-isolation. If, however, mesoderm is included, an aggregate is formed which holds together.

Townes (unpublished) has extended Holtfreter's work to disaggregates of embryonic amphibian cells. Isolates of ectoderm and endoderm are disaggregated with alkali, and the two cell types are randomly distributed within the resultant heap. In about 4 to 8 hours the cells have reaggregated, forming a sphere with a smooth exterior. At 72 hours, ectoderm and endoderm cells have sorted out to form essentially 2 aggregates, the one of ectoderm and the other endoderm, in close opposition. At 96 hours a further separation has taken place and the 2 aggregates are usually joined by a short narrow bridge.

If, on the other hand, portions of ectoderm, endoderm, and mesoderm are dissociated together it is found that, at the end of 3 to 8 hours, the cells have formed a smooth sphere, such as is obtained with ectoderm and endoderm cells above. At 72 hours the ectoderm cells have migrated to the surface of the mass and appear as patches among endoderm cells. If sufficient ectoderm cells are supplied, they will form a covering for the entire mass. The majority of the mesoderm cells lie immediately under the ectoderm, between it and the endoderm, with a few cells scattered throughout the endoderm. The ectoderm does *not* separate from the endoderm to form two distinct aggregates, as it does in the absence of mesoderm and, at 96 hours, neural folds have formed in some cases.

TABLE 2

SUMMARY OF EXPERIMENTS SHOWING EFFECTS OF FROG ANTIGASTRULA SERUM ON REAGGREGATION OF DISSOCIATED FROG CELLS

	Mixtures of disaggregated ectoderm and endoderm cells		Mixtures of disaggregated ectoderm, mesoderm, and endoderm cells	
	Normal serum	Antiserum	Normal serum	Antiserum
Time (hours)	17	28	8	14
Aggregates rounded:	+	−	+	−
Exposed cell surfaces are flattened:	+	−	+	−
Random intermixture of ectoderm and endoderm cells:	−	+	−	±
Time (hours)	65	65	36	36
Ectoderm and endoderm in separate aggregates:	+	−	−	−

A few preliminary experiments have been carried out with antisera produced against *Rana pipiens* gastrulae. Its effect on various combinations of dissociated *Rana pipiens* cells were noted and the results are shown in TABLE 2. Experimental details have been published elsewhere (Spiegel, 1954b).

Effect of Antiserum on Disaggregated Ectoderm and Endoderm Cells of Rana pipiens

Ectoderm and endoderm cells of stage 11, after one hour in *normal* serum, had adhered and the surface was becoming smooth. By four hours, the aggregate had rounded up, and ectoderm and endoderm cells had sorted out. The cells at the surface were flattened, and a smooth exterior was noted at 17 hours. By 65 hours, the ectoderm and endoderm formed two separate aggregates connected by a short stalk of cells.

After 3.5 hours in antiserum, approximately 100 disaggregated ectoderm and endoderm cells, from a stage 11 embryo, adhered to each other, but the aggregate failed to round up. At 4 hours, the cells remained spherical, and the aggregate surface was not smooth. Ectoderm and endoderm cells appeared randomly dispersed. No slime was noted. At 65 hours, the ectoderm and endoderm cells still had not sorted out, and the surface was unchanged.

Effect of Rana pipiens *Antigastrula Serum on Disaggregated Ectoderm, Endoderm, and Mesoderm Cells of* Rana pipiens

After one hour in antigastrula serum, about 100 disaggregated mixed ectoderm, mesoderm, and endoderm cells, from a stage 12 embryo, had partially adhered. The ectoderm and endoderm cells appeared to have sorted out, but were loosely packed. No change was noted after this period. Reaggregation in normal serum was as previously described by Townes (unpublished) in Holtfreter's solution.

That the *Rana* antigastrula serum is specific is demonstrated by the ability of cells of *Triton alpestris* embryos to reaggregate normally in this antiserum.

The results with embryonic amphibian cells are difficult to explain except

in terms of the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces similar to those between antigens and homologous antibodies. As a logical consequence of this hypothesis, the segregation of ectoderm and endoderm cells from each other in normal serum into two separate aggregates would indicate that different surface antigens are present on each of the two cell types. Recent evidence obtained by Clayton (1953) with *Triton alpestris* embryos seems to indicate that ectoderm and mesoderm, at least, contain different antigens. Endoderm antigens, however, were not investigated, and the location of these antigens in the cell was not determined.

The fact that mixtures of ectoderm, endoderm, *and* mesoderm cells, in Holtfreter's solution or normal serum, round up to form a single aggregate consisting of three concentric layers of cells, with the mesoderm layer situated between ectoderm and endoderm suggests an interesting possibility with regards to the surface antigens of mesoderm. Perhaps mesoderm, in addition to possessing specific surface antigens which are important for the adhesion of mesoderm cells to each other, also possesses sites which are complementary to ectoderm surface antigens *and* to endoderm surface antigens. Thus, in effect, mesoderm would act as a heteroligating antibody to ectoderm cells and to endoderm cells, and would be capable of binding the two cell types together. This action would be analogous to the hypothesis proposed for the failure of segregation of mixtures of the cells of two species of sponge in antiserum produced in rabbits by simultaneous injection of cells of both species. Here it was suggested that *the rabbit* formed heteroligating antibody.

Final Conclusions

It seems superfluous to assume some other mechanism of cell adhesion in amphibians than in sponges. In amphibia, however, there are two complicating factors—the apparent importance of calcium in reaggregation, and the success of xenoplastic transplants.

In a calcium-free medium, amphibian cells will not reaggregate (Holtfreter, 1943), and portions of tissue dissociate into single cells. On the other hand, the segregation of *Triton* ectoderm and endoderm cells in *Rana* antigastrula serum, in contrast to the nonsegregation of *Rana* cells, indicates that specific surface antigens are essential to adhesion, at least in *Rana*. The apparent contradiction is reminiscent of the double mechanism of reaggregation shown by the transitory association of *Microciona* and *Cliona*. Of course, in this case, the initial association can scarcely depend on calcium, but it may involve some comparable nonspecific agent.

It may be that the only difference between amphibia and sponges is that, in sponge, the specific forces are strong enough to pull cells together in opposition to the nonspecific forces; in amphibia, the specific adhesive forces, in early stages, are too weak to hold cells together by themselves and must be supplemented by the nonspecific action of calcium. This difference would account for the initial success of early xenoplastic transplants. The failure of such transplants, when made at later stages, would seem to indicate that surface antigens become increasingly important as development proceeds. In that

case, however, one might ask why an early xenoplastic transplant does not fail later.

Two answers seem possible: (1) by the time the surface antigens assume a major role, the transplant may be held in place by other, and still stronger, forces, such as overlying tissue layers; (2) it may be possible that the nature of the later-formed surface antigens at the boundary between host and donor tissue is somehow modified by the contact so as not to generate repelling forces; this suggestion points to a possibly interesting type of experiment.

The Tyler-Weiss hypothesis can also allow us to make certain suggestions regarding the sustained output of cells, from various germinal tissues, *i.e.* lymphocytes, and for the metastases which occur in certain tumors. A particular antigen or autoantibody (not necessarily in the serum) may be lost, or its rate of formation decreased, during the formation of the lymphocytes. The cells can no longer adhere to each other and now exist, in effect, as a suspension of cells. The same thing may be true for erythrocyte formation. Coman (1953) has suggested that a possible mechanism for the origin and distribution of blood-borne tumor metastases may be a lowered adhesiveness of cells related to a deficiency in calcium. An alternative or concomitant explanation would be the loss of some antigen or autoantibody.

The sponge, amphibian, and erythrocyte experiments have also led us to certain conclusions which are of more than passing immunological interest. The demonstration of heteroligating antibody molecules in rabbit antiserum allows us to conclude, if the present theory concerning antibody synthesis is correct, that during the foldings of the polypeptide chain of the antibody molecule, both ends of the chain are *not* always influenced by the same antigen and, perhaps more frequently than is known, can be influenced by *two different* molecules.

The relation of antibody synthesis to normal globulin formation and to protein synthesis in general has been pointed out by many authors. We are hopeful that, in time, future experiments will not only offer some information concerning the problems of selective cell adhesion but may also bear on certain problems of protein synthesis.

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Discussion of the Paper

Question: Have you studied the effects of an electric field on reaggregation?

Doctor Spiegel: We have carried out a few experiments with *Microciona* cells and have obtained the following tentative results. Reaggregation is inhibited at the anode and appears to be normal or even enhanced at the cathode. The few counts that we have made indicate a lower cell density at the anode than at the cathode, suggesting that the cells have migrated towards the cathode. However, our results are based on only two experiments.

Doctor Ernst W. Caspari: I thought that I noted in your recent paper that you observed a difference in the reaggregation of ectoderm and endoderm cells.

Doctor Spiegel: No, we did not. In this connection, however, I might mention the striking reorganization of granules that occurs in the amebocytes of sponges during reaggregation. Prior to reaggregation, the granules are located at random within the granuloplasm. As soon as two cells adhere to each other and the hyaloplasms fuse, the granules reorient so as to form a shell at the periphery of the granuloplasm.

Doctor Ernst W. Caspari: Have you attempted to stir up the granules of a cell with a needle or by introducing a nickel particle and then stirring with a magnet? This procedure would allow one to see if this granule reorganization is reversible and, if so, how often it can be repeated.

Doctor Spiegel: Sponge cells vary in diameter from about 4 microns (the collar cells) to about 15 microns (the amebocytes). It does not appear to be technically feasible. We have tried tearing aggregates apart with glass needles and the cells will now reaggregate again, apparently in normal fashion. In this type of experiment, we could very easily see if the granules will again become located at random in the granuloplasm and then reorient.

Doctor Wilson: Do you think that calcium can be rejected as playing any role in the reaggregation of cells?

Doctor Spiegel: No. It is well known that calcium plays a role in the adhesion and reaggregation of other cells as, for example, embryonic amphibian cells, sea-urchin blastomeres, *etc.* On the basis of the evidence that we have presented, it does not seem to play any role in the reaggregation of sponge cells. Perhaps sponges are unique in that calcium is not needed for normal cell adhesion.

Doctor Clifford Grobstein: I do not think that simply not being able to see any slime or cement is sufficient evidence to reject its possible presence.

Doctor Spiegel: De Laubenfels reported such a slime to be present in the sponge *Iotrochota birotulata*. He believes that the contraction of this slime is of greatest importance in reaggregation and not ameboid movement. We have studied the reaggregation by means of time-lapse photographs and have not been able to see any slime contracting. We have also used various stains in order to detect a slime or cement and have not been able to see any evidence of it in the forms studied. By moving a glass needle through a suspension of cells, it can be noted that the cells move independently of each other when stirred in this manner.

Doctor Grobstein: From what stage in *Rana pipiens* embryos did you isolate ectoderm and endoderm?

Doctor Spiegel: Stages 11 and 12.

Question: Is it possible that the specific adhesion is due to a difference in the surface tension of each cell species? Have you tried using detergents?

Doctor Spiegel: No, we have not tried using detergents. I frankly think that it would be much more difficult to interpret the results in terms of differences in surface tensions. I doubt very much that one could very easily explain the formation of mixed aggregates in the mixed antiserum in these terms But, as you say, it would be very easy to try several detergents. What we are suggesting as a possible mechanism of adhesion may not be entirely correct but, on the basis of the experimental results, we think that it is the most probable explanation and are proceeding to test this hypothesis further in future experiments.

Doctor A. M. Schechtman: Could the antisera be acting on some site on or in the cell other than the actual adhesion site and thus affect enzymes, *etc.*, and inhibit reaggregation?

Doctor Spiegel: That is a very important point that you have raised, and I am afraid it cannot be answered at the present time. You are correct when you state that antibody may be directed against some constituent of the surface that is not directly concerned in reaggregation. Such bound antibody might partly overlie the chemical sites actually involved in adhesion. We cannot, at present, distinguish this possibility from the possibility that the antibody directly combines with the adhesive sites.

Doctor A. M. Schechtman: Have you noted any differences in cell movement, shape, *etc.*, after the addition of antiserum?

Doctor Spiegel: No. The amebocytes move at about the same rate as in normal serum, and the pseudopodial shape and length are unchanged. The collar cells are also not affected visibly. The cells lose their ability to adhere

to each other, but all other characteristics are unchanged, as far as we can tell at the present time.

Doctor Schechtman: Have you tried placing the cells in an inert viscous medium?

Doctor Spiegel: No.

Doctor Ernst W. Caspari: Do you know when these surface antigens first appear in development?

Doctor Spiegel: In sponges, of course, we are dealing with adult cells, and so these antigens are probably present before disaggregation. We have not studied the time of appearance of surface antigens in embryonic amphibian cells, but that is a future project.

THE CELL NUCLEUS IN RELATION TO THE PROBLEM OF CELLULAR DIFFERENTIATION

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With the development of genetics and experimental embryology came the realization that two different sets of factors must be involved in embryonic differentiation. The intranuclear units of genetics have been shown to play a role (although how extensive is debatable) in the development of a great variety of morphological and physiological features at every stage in the life history of the organism. On the other hand, the analysis of developmental forces by experimental embryologists could proceed as if genetic factors did not exist or were of minor consequence in the elaboration of the basic tissues and organs. To the embryologist, it was clear that localized cytoplasmic differences, whether in the zygote or in later multicellular stages of the organism, and irrespective of their own origin, were more immediately concerned with the further course of differentiation.

Meanwhile cytological observations on the individuality of chromosomes and their behavior in cell division lead to a very plausible conclusion, namely, that the genetic factors must be equal or homogenous in the somatic tissues. Certain reservations were required, however, since genetic factors obviously do not express themselves at the same time in development nor do they operate homogenously in all the cells of the organism. It was necessary, therefore, to qualify the concept of nuclear homogeneity. One must assume that (1) the genetic factors are modified or otherwise made dissimilar in various differentiated cells, whether such modification is governed by intranuclear or other factors; or (2) that the genetic factors retain their original nature and only their function is modified, activated, or depressed, probably by the specific cytoplasmic environment of each nucleus. As is well known, experimental embryology provided observations which indicated that given cytoplasmic regions carry out their usual differentiations irrespective of the particular nuclei which may reside in these regions. Although such observations tell us more about the cytoplasm than about the nucleus, the second of the above assumptions gained broad although tentative acceptance as a generalized theory of development.

The difference between the two theories is a subtle one not readily subject to experimental verification. The closest approach to verification was a series of experiments which led to the rejection of the Roux-Weismann theory of differentiation, with its postulate of differential distribution of genetic factors during cell division. These experiments, and others since then, showed that, in several invertebrate and vertebrate organisms, the nuclei of the very young organism, or at least some of them, may be redistributed to different cytoplasmic regions without appreciable alteration of development. This phenomenon does not establish the homogeneity of even the earliest nuclei of the embryo. It is possible, for example, that the nuclei are already different in these early stages but that, in common with some regions of the embryo (of which, after

all, they are a part), they are capable of adapting or regulating with reference to new cytoplasmic environments in which they find themselves. Stated otherwise, the nuclei and especially the early nuclei may undergo reversible modification which becomes stable or irreversible in later development and, possibly, at different times in different tissues. In the absence, however, of supporting evidence for this possibility, we are justified in the assumption that at least some of the early nuclei of the organism are developmentally homogeneous or equal.

The probable equality of the early nuclei is consistent with a substantial and well-known body of evidence concerning the control of early development, namely, the predominance of maternal influences. Early events of development seem, to a large extent, to be governed by prior directives originally set up in the ovum during its period of tenure in the maternal body. Sooner or later, the action of the paternal genetic factors becomes evident, and we may assume the action of the maternal factors is also delayed to corresponding times. It may be, then, that intranuclear factors are developmentally quiescent during early stages (the "silent period" of Zeuthen) and their functions limited to the nondevelopmental aspects of cell metabolism and division. Each of the early nuclei may well be an exact replica of the zygote nucleus during this period. The situation, however, may be quite different when immediate gene action (as distinct from prior maternal genetic factors) becomes apparent and is differentially exerted in various tissues. The developmental equality of such nuclei, to my knowledge, has not been studied. In the words of Stern (1954), "How far are we permitted to extrapolate from the proofs for full potency of the nucleus in its early stages to the assumption that the omnipotency still exists after *full* differentiation?"

The most recent work on developmental equality of early nuclei is that of Seidel (1952) on the rabbit, and of King and Briggs (1954) on the frog. In the latter, some of the nuclei, removed together with some cytoplasm from the archenteric roof or presumptive neural plate of advanced gastrulae, were injected into enucleated zygotes and they proved capable of replacing the zygote nucleus in further development. The failure of some eggs to develop and the abnormal development of others were attributed to injuries inflicted during transplantation. More recently, the same authors have obtained results which indicate that abnormalities or failure to develop may not be attributed entirely to operational injury but may actually depend upon developmental inequality of nuclei (personal communication).

In certain aspects, the inequality of nuclei in differentiated tissues is obvious. Visible morphological and size variations of nuclei within the organism are common knowledge. Such variations are consistent features of various cells and, from the practical point of view of tissue identification, among the characteristic features of differentiation. Some remarkable changes have been observed in the size of nuclei after injury, transplantation, ligation, *etc.* (see Altschul, 1948). A great deal of size variation is attributable to polyteny, polyploidy, and other factors which have been discussed by Fankhauser (1954). Differences in heterochromicity as well as polyploidy have been considered by Huskins (1947) from the point of view of possible correlations with differen-

tiation. Even the quantitative constancy of DNA per nucleus of a given species, considered by many investigators to be one of the most invariable features of somatic as well as germ cells, has not yet been established (compare, for example, Davidson, 1953; Lison and Pasteels, 1950; Pasteels and Bullough, 1953).

Nuclear changes during the course of development are common and, in some instances, have been correlated with other morphological and physiological differentiations or with growth and protein synthesis. The work of Caspersson and his associates (Caspersson, 1950) has demonstrated nucleolar variations associated with growth, differentiation, and protein synthesis. Flexner (1954) has described changes in nuclear growth and in the nucleolus of neuroblasts. He has also described the close correlation of these changes with changes in respiratory and glycolytic enzyme activity, the sprouting of cell processes, and the rapid elaboration of Nissl substance.

Such dissimilarities in nuclei, whether they be designated "maturation" or "differentiation," are certainly too crude to represent causal factors responsible for the intricacies of differentiation. They seem to be expressions of the *nondevelopmental functional operations* of the nucleus, in spite of the fact that certain of the changes are closely associated with differentiative processes. But we cannot be certain. The occurrence of such dissimilarities and the probability that nuclei will be shown to differ in many chemical aspects such as enzymatic content, proportion of DNA in relation to other constituents, quantity of residual chromosome protein, quantity and nature of lipids, *etc.*, raises the fundamental question as to which component(s) of nuclei represent the carriers of genetic factors. These components, of course, would be the critical ones to investigate, with the aim of gaining insight into the problem of nuclear equality or inequality, and thereby a better understanding of fundamental processes of differentiation. Although there is no certainty as to the chemical nature of the genetic (developmental) factors, an impressive if not conclusive body of evidence points to desoxyribonucleic acid or a complex closely associated with it.

The importance of the chemical and serological approaches to the problem of genetic equivalence of somatic nuclei, and the simultaneous extension of nuclear transplantation, cannot be emphasized too strongly. Each approach of itself is likely to reach a theoretical impasse. Let us suppose it proves impossible to obtain well-developed organisms after transplantation of nuclei from differentiated tissues, whereas identical technique is frequently successful with nuclei from the very young embryo. This supposition seems a distinct probability from the current work of Briggs and King. Such results will indicate that the older nuclei are *not equivalent developmentally. They will not show that genetic factors differ* in differentiated tissues, which is the critical point for our concept of general mechanisms of differentiation. Specializations for nuclear nondevelopmental functions, relationships between nuclei and specific cytoplasmic functions, may prevent successful transplantation. Similarly, if DNA is eventually shown to be tissue-specific, as seems to me most probable in view of the possibilities for variation in such a molecule, we shall not be able to conclude that nuclei are genetically equivalent, since the differences in DNA

may here, too, be incidental to nondevelopmental function and without effect upon the hypothetical genetic function. Correlation of the two approaches, preferably with the use of the same species, greatly increases the possibilities of incisive analysis.

The Stedmans (1947) have suggested that only proteins are "capable of accounting in a broad manner for the hereditary functions of the chromosomes." However, on the basis of the double helical structure of desoxyribose nucleic acid (DNA) proposed by Watson and Crick (1953), DNA may exist in an enormous number of isomeric forms depending upon the order of the bases bridging the coils of the two helices (see also Crick, 1954). We have no reason, therefore, to discard, on chemical grounds, the nucleic acids or nucleoproteins as the essential structures which many geneticists and cytochemists tentatively believe them to be. If the problem of nuclear equality or inequality is to be subject to any tests other than nuclear transplantation or redistribution (which, as stated above, may turn out to be inconclusive because of disturbances in nondevelopmental functions of nuclei), then we are certainly justified in focusing our first attentions upon the chemical evidence for DNA equality or inequality in differentiated tissues.

DNA from a variety of species and tissues shows, as is to be expected, a number of similarities and dissimilarities. The situation is complicated by differences in preparation, and the strong probability that varying degrees of contamination, recombination, and denaturation doubtless enter the picture. There seems to be a high degree of similarity in such aspects as elementary composition, viscosity, electrophoretic mobility, and X-ray diffraction pattern. Possibly there are real differences in absorption centers for ultraviolet, the depolymerizing effects of acids, and in molecular weight (Zamenhof and Chargaff, 1950; Watson and Crick, 1953; Wilkins, Stokes, and Wilson, 1953).

Chargaff and his associates (Chargaff, 1950) discern two broad categories of DNA: an AT type, characterized by the quantitative predominance of adenine and thymine, and a GC type, in which guanine and cytosine predominate. The former seems to be characteristic of animals and of certain viruses, the latter of plants, including bacteria. Wyatt (1950) has evidence that bacterial DNA may be distinguishable from that of higher animals and plants by the absence of 5-methylcytosine, and Smith and Wyatt (1951) conclude that the DNA of *Escherichia coli* is transitional.

Chargaff, Lipshitz, and Green (1952) studied the pentose and the bases derived from sperm DNA of four species of sea urchins. The sugar could not be distinguished from that found in thymus DNA, and ultraviolet absorption was similar. On the basis of the molar ratios of adenine to guanine, and of thymine to cytosine it was possible to distinguish one species (*Arbacia lixula*) from the other three (*Psammechinus miliaris*, *Echinocardium cordatum*, and *Paracentrotus lividus*). Smith and Wyatt (1951) could distinguish the DNA of two closely related bacteriophages from that of the host organism (*Esch. coli*). Chargaff and his associates (1950, 1952) sum up their experience in the statement that DNA is probably characteristic of the species.

Analyses of molar proportions of bases have not permitted demonstration of

organ- or tissue-specific DNA (Chargaff, 1950a). Preparations from human sperm, thymus, liver, and a cancer originating in the colon were indistinguishable (Chargaff, 1950b). Griffin and Rhein (1950) found that the molar ratios of liver DNA were altered during azo-dye carcinogenesis and also in the tumors produced. The carcinogen acetylaminofluorine had no such effect, however, so that it is doubtful that DNA alteration is associated with the establishment of the neoplastic state.

It is, of course, possible that different approaches will reveal dissimilarities. Thus Korngold and Pressman (1952) reported that antiorgan sera (*e.g.* anti-lung) could be made more specific for the homologous organ by absorbing the antisera with homologous nucleohistone or with the DNA fraction. The absorbed antibody was eluted and studied for its capacity to localize in various organs. However, the residual chromosome fraction (which remains after most of the DNA is washed out) gave better antibody purification than the DNA. Together with quantitative work on the varying proportions of residual material in chromosomes from different tissues (see, for example, Mirsky and Ris, 1947), this finding suggests that a high degree of tissue specificity may reside in complex protein fibers of the nucleus.

It should be noted that ribonucleic acid (RNA) from different species also shows variations in molar ratios of its bases. Of still greater importance, some evidence (Chargaff *et al.*, 1950; Davidson, 1953), indicates pronounced organ-specificity of RNA within a given species, and at different developmental stages. Thus, calf liver, spleen, and thymus seem to have quite distinct RNA's as do pig pancreas and liver; and calf liver RNA has been distinguished from that of beef liver.

The usefulness of serological methods in distinguishing *Pneumococcus* polysaccharides, serum proteins of closely-related species, and even proteins within a species (see, for example, Marshall and Deutsch, 1951; Ebert, 1953) suggests that such methods should be of value for studies of nuclei and nucleic acid-containing substances.

The early work on fractions containing nucleic acids involved preparations which were highly denatured, and the results indicate little more than that the specific preparations used were toxic but not antigenic, or antigenic but probably not organ-specific (Beebe, 1905; Pearce and Jackson, 1906; Frank, 1907; Feissinger, 1909; Pearce, Karsner, and Eisenbrey, 1911; Wells, 1913).

Sevag, Lackman, and Smolens (1938) found nucleic acids can combine specifically with antibody. A nucleic acid fraction, isolated from water-insoluble bacterial residue, reacted in precipitin tests with antiserum against homologous type *Streptococci*. Lackman *et al.* (1941) obtained horse antipneumoccus sera and found them to be specifically reactive with nucleic acid preparations from thymus, sperm, yeast, and *Streptococci*. The antisera contained antibodies specific for nucleic acids, since they could be absorbed with pneumococcus carbohydrate and yet retain their activity for nucleic acids. On the other hand, absorption with a single nucleic acid preparation, such as thymus nucleic acid, removed all or nearly all activity for the four nucleic acids, leaving antibodies for pneumococcus carbohydrate. It is to be noted that the antisera

were prepared against the entire bacteria, not against the nucleic acid fraction. Rabbits usually gave antisera incapable of reacting with the nucleic acids, although horse antisera were successful.

Our own experience with antisera against nuclear constituents followed this plan: nuclei were isolated from organs or tissues of three species of birds of known relative taxonomic position, the chicken and turkey (Galliformes), and the duck (Anseriformes). It is clear from the work of Landsteiner and van der Scheer (1940), of Boyden and his associates (1942, 1943), and others, that the relative taxonomic positions of these and other species is readily demonstrable by means of rabbit antisera directed against the serum or egg-white proteins. If (1) the correct relative taxonomic relationships could be duplicated by means of antinuclear sera, and (2) the nuclear antigens used could be shown to be nucleus-specific (*i.e.*, without activity for cytoplasmic materials and serum), then the antisera might be considered reliable, and could be used to test the equality or inequality of nuclear constituents from several organs.

Liver nuclei from the three species were obtained by a slight modification of the Dounce method (1943), using citric acid solutions followed by distilled water of controlled pH. Essentially the same method was used for spleen nuclei, but with alterations of centrifugal time and speed necessitated by the smaller size of the nuclei. Red blood cell nuclei were prepared by the saponin method of Dounce and Lan (1943). To make all preparations as similar as possible, portions of the liver and red cell nuclei, after completion of the Dounce procedure, were treated with saponin. We may dispose of the latter nuclei at this time, since they gave no serological reactions distinguishable from those obtained with nuclei not treated with saponin.

The red cell nuclei were contaminated with stromata to varying degrees, a difficulty noted by Dounce and Lan (1943) and Laskowski and Ryerson (1943). The liver nuclei were clearly less contaminated with cytoplasmic material than those from spleen and red cells. For serological purposes, however, it is probably safer to assume that all nuclear preparations are contaminated sufficiently to influence antibody production, and to take measures accordingly. In the present instance, we absorbed the antisera with various cytoplasmic fractions.

From a few preliminary experiments in which whole nuclei were injected, we gained the impression that whole nuclei are not good antigens. Nuclei were therefore first extracted with 10 per cent saline, and the entire material, including both soluble and insoluble components, was dialyzed against 0.9 per cent saline. Having reduced the salt concentration, the entire contents of the dialysis tube was used as injection antigen. Rabbits were given graded intraperitoneal and intramuscular injections on alternate days, and were bled 10 days after the last injection. Two extracts were prepared from each nuclear preparation: (1) a fraction soluble in 10 per cent NaCl solution but not in 0.9 per cent saline, and (2) a fraction soluble in 0.9 per cent saline as well as 10 per cent saline.

The 10 per cent saline removes 90 to 92 per cent of the mass of chromosomes (Mirsky and Ris, 1947) including most of the DNP. It probably also removes small amounts of phosphoprotein which seem to be present in nuclei, some RNP, and a miscellany of other substances. On dialysis against 0.9 per cent saline,

TABLE 1

ANTISERA AGAINST LIVER NUCLEI FROM THE CHICKEN, TURKEY AND DUCK REACTED WITH 0.9 PER CENT SALINE SOLUBLE FRACTION OF LIVER NUCLEI*

Antigen (from liver of)	Antisera vs. liver nuclei from		
	Chicken	Turkey	Duck
Chicken	8–1/75	5–1/15	0
Turkey	5–1/15	7–1/50	3–1/5
Duck	0	0	10–1/50
Rat	0	0	0
THE SAME ANTISERA REACTED WITH 10 PER CENT SOLUBLE FRACTION OF LIVER NUCLEI			
Chicken	5–1/15	5–1/15	3–1/15
Turkey	5–1/15	4–1/10	9–1/100
Duck	0	0	6–1/25
Rat	0	0	0

* First number in column is tube number, followed by dilution value. Undiluted antigen, 8 mg./ml. Thus 8–1/75 indicates a positive reaction in all tubes through the eighth, which contains a dilution of 1:75.

threads composed largely of DNP appear (Mirsky and Pollister, 1946). These threads were washed with 0.9 per cent saline, then redissolved in 10 per cent saline, and are here designated the 10 per cent saline soluble fraction. The fraction soluble in 0.9 per cent saline contains RNA as well as globulinlike substances.

Except for the initial treatment with citric acid, the preparations were maintained in the pH range 5.8 to 7.0 and at temperatures close to 3 to 5°C. or lower. Even mild acids and bases tend to disintegrate nucleic acids to smaller units with a decrease of viscosity and streaming birefringence (Gulland, 1947).

TABLE 1 shows that antisera against liver nuclei show clear species specificity when reacted with both nuclear fractions and without any preliminary absorption. The 0.9 per cent soluble fraction shows greater specificity than

TABLE 2

ANTISERA AGAINST RED BLOOD CELL NUCLEI FROM CHICKEN, TURKEY, AND DUCK REACTED WITH 0.9 PER CENT SALINE SOLUBLE FRACTION OF RED BLOOD CELL NUCLEI*

Antigen (from RBC of)	Antisera vs. RBC nuclei from		
	Chicken	Turkey	Duck
Chicken	10–1/150	10–1/150	5–1/15
Turkey	9–1/100	6–1/25	5–1/15
Duck	6–1/25	5–1/15	9–1/100
THE SAME ANTISERA REACTED WITH 10 PER CENT SALINE SOLUBLE FRACTION OF RED BLOOD CELL NUCLEI			
Chicken	9–1/100	7–1/50	7–1/50
Turkey	7–1/50	10–1/150	9–1/100
Duck	6–1/25	5–1/15	9–1/100

* First number of column is tube number, followed by dilution value. Undiluted antigen, 8 mg./ml.

the 10 per cent (DNP) fraction. An anomalous reaction is seen between antiduck serum and turkey antigen. We have noted on many occasions that turkey antigens yielded excessive precipitates (see also FIGURE 4).

Antisera against red cell nuclei show a degree of species specificity (TABLE 2) but anomalous reactions (see antiturkey and chicken antigen, antiduck and turkey antigen) suggests an uncontrolled variable which we believe to be the different amounts of stromata in both injection and test antigens. Laskowski and Ryerson (1943) found that degree of contamination with stromata shows no apparent correlation with technique. It is equally possible that other factors are involved. Thus, lipids are known to influence antigen-antibody reactions, and various nuclei show considerable variation in lipid content (Dounce, 1943; Williams *et al.*, 1945). Similar anomalous results were obtained with antispleen sera.

If anomalous reactions are due in part to cytoplasmic contamination, we might expect to obtain anomalous results also when the antisera against the nuclei of spleen and red cells are reacted with extracts of the cytoplasmic fractions from the three species. This reaction, in fact, was found, and the point was further verified by the consistent behavior of the antiliver sera with cytoplasmic fractions of liver (TABLE 3). This finding shows that, without any prior absorption, the antiliver sera show a high degree of species specificity. If we now compare TABLES 1 and 3, it is evident that the reactions of these

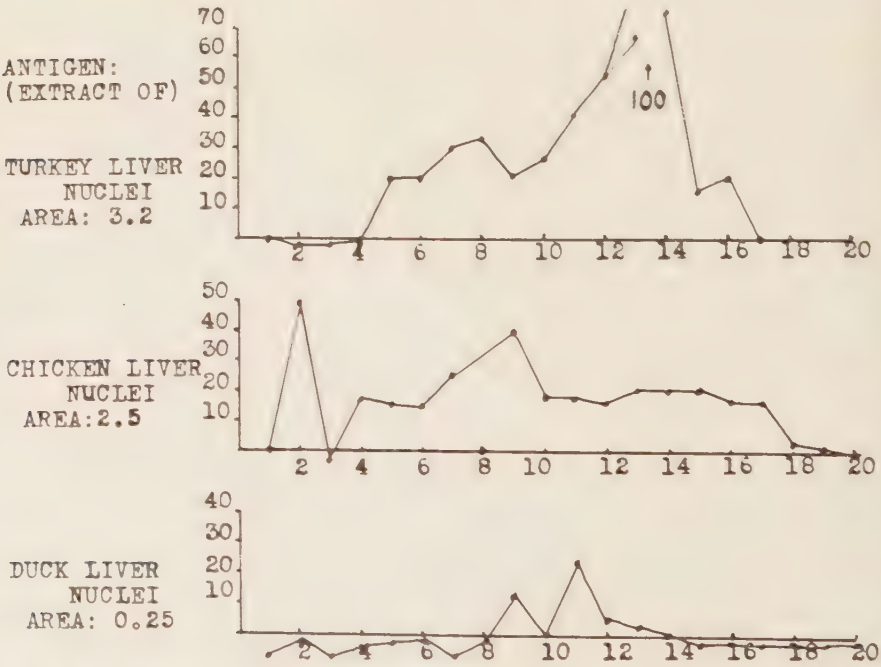

FIGURE 1. Antisera against nuclei from turkey liver reacted with nuclear extracts of several bird species (0.9 per cent saline soluble fraction).

TABLE 3

ANTISERA AGAINST LIVER NUCLEI REACTED WITH BLOOD SERUM AND CYTOPLASMIC FRACTON OF LIVER (0.9 PER CENT SALINE SOLUBLE)

Cytoplasmic fraction of	Antisera vs. nuclei from the livers of		
	Chicken	Turkey	Duck
Chicken liver	3	0	0
Chicken serum	+	0	0
Turkey liver	0	3	0
Turkey serum	+	+	0
Duck liver	0	0	4
Duck serum	0	0	0
THE SAME ANTISERA REACTED WITH CYTOPLASMIC FRACTIONS SOLUBLE IN 10 PER CENT SALINE			
Chicken liver	0	0	0
Chicken serum	+	0	0
Turkey liver	5	0	0
Turkey serum	0	+	0
Duck liver	0	0	0
Duck serum	0	0	0

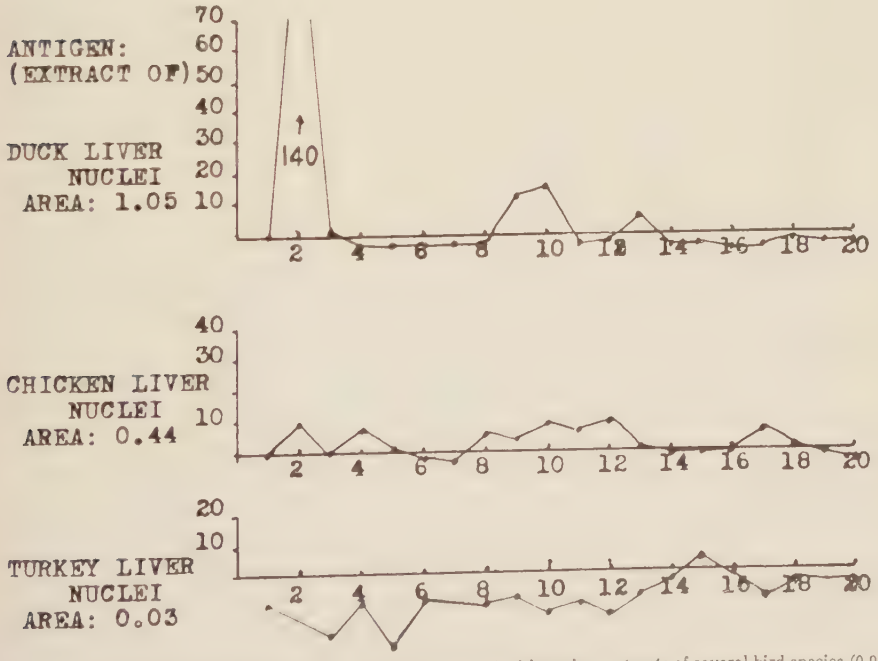

FIGURE 2. Antiserum against nuclei from duck liver reacted with nuclear extracts of several bird species (0.9 per cent saline soluble fraction).

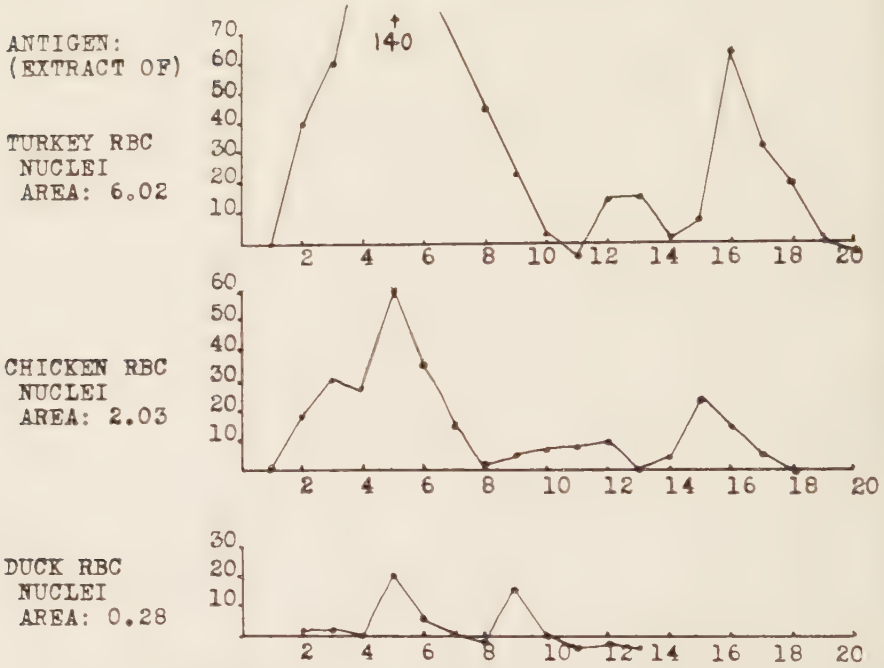

FIGURE 3. Antisera against nuclei from turkey red blood cells reacted with nuclear extracts of red blood cells (0.9 per cent saline soluble fraction).

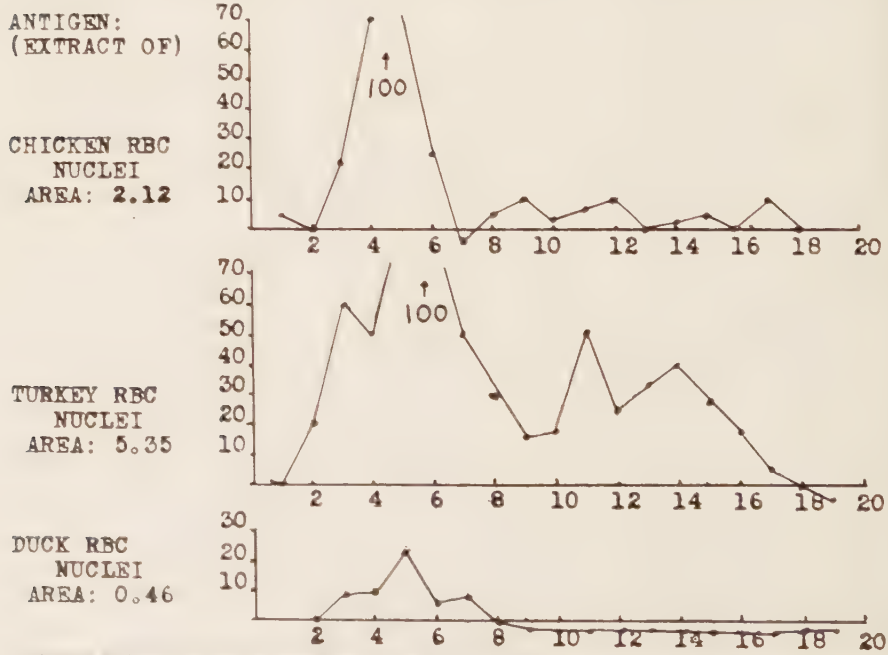

FIGURE 4. Antiserum against nuclei from chicken red blood cells reacted with nuclear extracts of red blood cells (0.9 per cent saline soluble extracts).

TABLE 4

ANTISERA AGAINST LIVER AND SPLEEN NUCLEI ABSORBED WITH A CYSTOPLASMIC FRACTION AND THEN REACTED WITH VARIOUS CYTOPLASMIC AND NUCLEAR FRACTIONS SOLUBLE IN 0.9 PER CENT SALINE*

	Antisera against the nuclei of				
	Chick. liver	Chick. spl.	Turk. liver	Turk. spl.	Duck liv.
Cytopl. (abs. agent) ...	Chick spl.	Turk. liv.	Turk. spl.	Chick liv.	Turk. spl.
ANTIGENS (CYTOPLASMIC FRACTIONS SOLUBLE IN 0.9 PER CENT SALINE)					
Chick.					
Liver.....	0 (+)	0 (+)	0 (0)	0 (+)	0 (0)
Spl.....	0 (+)	+ (+)	0 (0)	+ (+)	0 (0)
Turk.					
Liver.....	0 (0)	0 (+)	0 (+)	0 (+)	0 (0)
Spl.....	0 (0)	0 (+)	0 (+)	+ (+)	0 (0)
Duck					
Liver.....	0 (0)	0 (0)	0 (0)	0 (0)	+ (+)
ANTIGENS (NUCLEAR FRACTIONS SOLUBLE IN 0.9 PER CENT SALINE)					
Chick.					
Liver.....	+ (+)	+ (+)	+ (+)	+ (+)	0 (+)
Spl.....	+ (+)	+ (+)	0 (+)	+ (+)	0 (+)
RBC.....	0 (+)	+ (+)	0 (0)	+ (+)	0 (+)
Turk.					
Liver.....	+ (+)	+ (+)	+ (+)	+ (+)	0 (+)
Spl.....	+ (+)	+ (+)	0 (+)	+ (+)	+ (+)
RBC.....	0 (+)	0 (+)	0 (0)	+ (+)	0 (0)
Duck					
Liver.....	0 (0)	0 (+)	0 (0)	0 (+)	+ (+)
RBC.....	0 (0)	0 (+)	0 (0)	+ (+)	0 (+)

* Symbols in parentheses show the reaction of the antisera before absorption with the cytoplasmic fraction indicated.

antinuclear sera with heterologous nuclei cannot be attributed solely to cytoplasmic contamination. Thus, the antiturkey serum reacts specifically with turkey-liver cytoplasm but with both turkey and chicken nuclear materials. Antiduck serum reacts only with the 0.9 per cent soluble fraction of duck-liver cytoplasm but with the nuclear antigens from all three species. The antisera therefore contain *nucleus-specific* antibodies at least for heterologous species.

The existence of nucleus-specific antigens is further indicated by absorption of the antisera with various cytoplasmic fractions (TABLE 4). Thus, when antiserum against the nuclei of chicken liver is absorbed with spleen cytoplasmic fraction, activity for all the cytoplasmic fractions is eliminated. Yet the antiserum retains activity for the nuclear fractions of both liver and spleen, although not for the red cell nuclei.

It will be noted that antiturkey liver sera can be made even more specific by absorption with turkey-spleen cytoplasm. This antiserum was then capable of distinguishing liver nuclear antigens from those of spleen and red cells. In no other antiserum was such specificity observed. However, the unmistakable *trend toward more restricted antinuclear reactivity* for extracts from heterologous

TABLE 5

ANTISERA AGAINST NUCLEI FROM CHICKEN TURKEY AND DUCK TESTED AGAINST NUCLEAR EXTRACTS FROM THE HOMOLOGOUS SPECIES (FRACTION SOLUBLE IN 0.9 PER CENT SALINE)

Antigen-extracts of nuclei from	Antisera vs. nuclei from							
	Chicken			Turkey			Duck	
	Liver	Spleen	RBC	Liver	Spleen	RBC	Liver	RBC
Liver	8	11	10	7	12	9	10	8
Spleen	5	6	10	3	12	6	—*	—*
RBC	9	11	10	0	6	6	9	9
THE SAME ANTISERA TESTED AGAINST FRACTION SOLUBLE IN 10 PER CENT SALINE								
Liver	5	5	5	4	9	5	6	5
Spleen	6	7	9	4	14	9	—*	—*
RBC	5	9	9	0	5	10	3	9

* — indicates no test performed.

organs as well as species, suggests the possibility that completely specific antisera, reactive with only a single type of nucleus, may be obtainable.

Antispleen sera uniformly retain a high degree of cross-reactivity after absorption with liver cytoplasm (TABLE 4). This fact may actually be evidence in favor of nuclear tissue specificity. It may reflect the several types of tissues held in common by the spleen, liver, and blood cells. The spleen contains large numbers of red cells, and reticuloendothelial elements are abundant in liver and spleen.

The reactions shown in TABLE 4 were carried out with nuclear fractions soluble in 0.9 per cent saline. TABLE 5 summarizes the reactions of the various antinuclear sera with both the 0.9 per cent and 10 per cent soluble fractions of nuclei of *homologous* species. It seems clear that the fractions show no consistent organ- or tissue-specificity. When the antisera are absorbed with cytoplasmic fractions, their activity for the DNP fraction of various nuclei within the species is restricted, that is, antisera tend to become less cross-reactive with nuclei from heterologous organs. Complete specificity for nuclei from a given organ was not observed.

Perhaps the most logical means of obtaining specific antinuclear sera would seem to be by absorption of the antisera with various heterologous nuclear preparations (TABLE 6). The use of entire nuclei, however, emulsified with the antisera, removed all activity for *all* nuclear preparations. Most unexpectedly, rat-liver nuclei, which showed no cross-reactivity with antibird sera (TABLE 1), were as effective as bird nuclei in removing antibody activity. This finding does not seem to be attributable to nonspecific adsorption of antibodies since rat nuclei do not abolish antibody activity when they are added to antisera against the spleen and red cells of birds (TABLE 6). These results may be rationalized, but only with the aid of a number of assumptions which make

TABLE 6

ANTISERA AGAINST NUCLEI FROM LIVER, SPLEEN, AND RED BLOOD CELLS ABSORBED WITH VARIOUS LYOPHILIZED NUCLEI AND THEN TESTED FOR SPECIFICITY WITH NUCLEAR EXTRACTS

Antisera vs. nuclei from	Nuclei used for absorption	Antinuclear activity after absorption
Chicken liver	Chicken red cells	0*
	Turkey liver	0
	Rat liver	0
Turkey liver	Turkey spleen	0
	Chicken spleen	0
	Rat liver	0
Duck liver	Duck red cells	0
	Chicken liver	0
	Rat liver	0
Chicken RBC	Chicken liver	Chicken RBC?
	Turkey spleen	Chicken RBC?
	Rat liver	All bird nuclei
Turkey RBC	Turkey spleen	0
	Rat liver	All bird nuclei
Chicken spleen	Chicken liver	0
	Turkey spleen	0
	Rat liver	All bird nuclei
Turkey spleen	Turkey RBC	0
	Chicken spleen	0
	Rat liver	All bird nuclei

* After absorption all antisera were reacted with, 10 per cent saline soluble fraction of liver, spleen, and RBC nuclei from chicken and turkey and of liver and RBC nuclei from duck. Similar results with antiduck sera.

the explanation too laborious to carry conviction. It is clear, however, that absorption of antinuclear sera with whole nuclei seems to be inferior to absorption with cytoplasmic constituents in so far as specificity is concerned.

The nephelometric estimates of antigen-antibody reactions shown in FIGURES 1 to 4 are based on the methods of Boyden and his associates (Boyden, 1942; Boyden and DeFalco, 1943). Numbers on the horizontal coordinate represent tube numbers in which tube 1 contains 8 mg. dry weight of nuclear extract per ml. Tubes 2 to 20 contain dilutions of tube 1, so that tube 20 contains a 1/32,488 part of the solids present in tube 1. The vertical coordinate gives the nephelos readings and therefore represents comparative amounts of precipitates. The areas under the curves give reliable estimates of taxonomic relationships when serum is used as antigen.

From FIGURES 1 and 2 it may be seen that the areas under the curves also give consistent indications of the mutual relationships of the chicken, turkey, and duck when *nuclear* extracts rather than blood serum are used. In FIGURES 3 and 4, we see a typical instance of discrepancies encountered both in the nephelometric and interfacial tests. Thus antisera against the nuclei of turkey red cells (FIGURE 3) gave expected results: maximum amounts of precipitate with homologous nuclear extract, moderate amounts with chicken, and almost none with the duck. The antichicken sera, however, gave a higher set of readings with turkey nuclear extracts than with homologous material (FIGURE 4). Attempts to show *organ*-specificity by means of the nephelometric method

TABLE 7

ANTISERA AGAINST VARIOUS NUCLEAR PREPARATIONS REACTED WITH 0.9 PER CENT SALINE EXTRACTS OF NUCLEI: AREAS UNDER THE NEPHELOMETRIC CURVES. COMPARE VERTICAL COLUMNS ONLY

Nuclear extracts of	Antisera against the nuclei of							
	Chicken			Turkey			Duck	
	Liver	Spleen	RBC	Liver	Spleen	RBC	Liver	RBC
Chicken								
Liver	2.30	0.30	1.01	2.51	3.62	2.15	0.44	0.61
Spleen	2.50	6.65	2.66	1.94	2.51	4.59	*	1.51
RBC	1.49	1.94	2.12	1.75	0.96	2.03	0.10	2.07
Turkey								
Liver	1.80	2.81	1.14	3.20	4.73	3.31	0.03	1.13
Spleen	3.34	1.32	1.91	2.65	2.53	1.72	0.45	1.84
RBC	0.02	1.17	5.35	2.55	1.80	6.02	0.55	2.73
Duck								
Liver	1.12	0.38	0.69	0.25	0.76	0.80	1.05	1.05
RBC	1.74	0.28	0.46	*	0.65	0.28	1.37	1.28

* No reaction observed in both ring and nephelos tests.

were completely negative, in the sense that the amount of variability abolished the significance of the instances in which consistent areal relationships were obtained (TABLE 7).

It is probable that the nephelometric method will prove more useful to the present problem when the antigens are brought to a greater degree of uniformity. The greatest value of this method has been with sera or serum fractions which contain relatively uniform types of proteins. Even with the use of sera, one may obtain anomalous results if chemical composition departs radically from that of the adult serum. We had a good instance of this anomaly in comparing embryonic and adult chicken sera (Schechtman, 1952). The embryonic serum is different from the adult in many respects, including a much greater proportion of lipids and lipoproteins as percentage of the total solids (Schechtman, 1952; Heim, 1954; Schjeide, 1954).

The nephelometric results, like the interfacial (ring) tests, were usually consistent when the antigens employed were fairly uniform and almost free from cytoplasm.

The remarkable cross-reactivity of antinuclear sera with cytoplasmic and serum constituents has been reported previously (Schechtman and Nishihara, 1950). We assumed that this action was due partly to contamination and partly to similarity of antigenic determinants in the nucleus, cytoplasm, and serum. Anderson (1953), however, says that bovine serum albumin, hemoglobin, and partly hydrolyzed gelatin may be able to penetrate into isolated nuclei. Roberts and Anderson (1951) observed that isolated nuclei fade on treatment with heparin. The nuclei seem to exchange a good deal of their nucleic acid for heparin. Gitlin, Landing, and Whipple (1953) used fluorescein-labeled antisera prepared against human serum proteins and found that nuclei in a number of excised human tissues combine with the labeled antibody.

Their results suggest that serum albumin, lipoprotein, and other serum proteins may normally be present in nuclei. The results are also interpretable in a different manner: the nucleus contains antigenic determinants like those of the serum proteins, not the proteins themselves. Coons, Leduc, and Kaplan (1951) injected heterologous serum proteins into mice and tested frozen sections with specific antisera. The foreign proteins were detected not only within cells but within nuclei of a variety of cells. It is of interest that hepatic parenchyma cells as well as reticuloendothelial elements seemed to take up the foreign proteins.

Summary

The concept that the nuclei of differentiated somatic tissues are equal as to their genetic factors (also referred to as nuclear genetic equivalence, intrinsic equality) has not been substantiated sufficiently. Experimental work on the redistribution or transplantation of early nuclei of the organism offer insufficient proof because of the possibilities (1) that early nuclei, like early embryonic cells, may be capable of regulation, or (2) that they have not yet differentiated and are concerned with nondevelopmental functions in the early maternally-dominated period of development.

Experimental verification of the theory of nuclear genetic equality is complicated by morphological and physiological changes which appear in various nuclei during development. Even if we assume that nuclear functions can be divided into two main categories, developmental and nondevelopmental, and that nondevelopmental functions operate in and around a framework of stable genetic factors, the two are so closely connected that separation is difficult.

Since DNA or DNA-containing substances seem most closely associated with genetic factors, some chemical and serological evidence on the specificity of various DNA preparations is reviewed. Both chemical and serological evidence favor the conclusion that DNA (or DNP) is probably different from species to species (in other words, they are species specific). The serological evidence also suggests that nuclei from remote species have antigens or antigenic determinants in common, and that nuclei contain antigenic determinants distinct from those in the cytoplasm (nucleus-specific antigen). Both of these conclusions are consistent with chemical data. Finally, neither the chemical nor the serological data are sufficient to allow the conclusion that DNA or any other macromolecular constituent of the nucleus is organ- or tissue-specific.

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Part III. Problems of Structural Organization

TISSUE DISAGGREGATION IN RELATION TO DETERMINATION AND STABILITY OF CELL TYPE

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Much information is available on the gradual stabilization of properties or developmental tendencies in regions and tissues of the embryo. Less secure information is available on the rise and stabilization of new properties in the component cells. Weiss (1939) has drawn a sharp distinction between "modulation" and true differentiation as two categories of induced cellular change. In the former category, he placed those changes which are system-dependent, *i.e.*, those changes which persist only so long as some extrinsic initiating influence is operating. In the latter category were put those changes which are system-independent, *i.e.*, those which are intrinsic and persist through successive cell generations in the absence of the extrinsic initiating influence.

Leaving aside, for the moment, the question of the general validity of the sharp distinction, it focuses our attention on the fact that in discussing "molecular events in differentiation" we are presumably referring, in present usage, to *system-independent cytodifferentiation*. In picking up signs of molecular change in development we should like to know how they relate to the precise time at which *cells* are biased or rigidly stabilized as to type. Most of our information on this point, however, represents extrapolation from the behavior of tissues or regions of the embryo explanted or transplanted to what are regarded as developmentally neutral sites. These classical tests for self-differentiative capacity have contributed to our appreciation of the rising stabilization of properties in the developing embryo, but we cannot safely assume that the source of the stabilization revealed lies in intrinsic cellular changes alone. Changes in cell number and density, boundary formation, intercellular matrix changes may all contribute to stabilization of properties in a cellular mass. It is a fact, though a melancholy one, that the only safe (but usually impractical) test for intrinsic, system-independent changes in cell type is the behavior of isolated cells. And it is also a fact, though an annoying one, that it is precisely in this isolated state that cells are most difficult to classify as to type.

Recently there has been a revival of interest, particularly with vertebrate material, in tissue disaggregation and reaggregation. Tissue dissociation and the subsequent restitution of body form have been studied in sponges and other lower invertebrates since the turn of the century, and the processes involved are not yet clear (Curtis, 1940). But, of late, many laboratories have been applying varied techniques for tissue disaggregation as an approach to problems of vertebrate tissue architecture, cellular differentiation, and embryonic induction (Del Pianto, 1942; Holtfreter, 1944; Moscona and Moscona, 1952; Grobstein, 1952; Weiss and Andres, 1952; Deuchar, 1953; Mookerjee, 1953;

Mookerjee. Deuchar and Waddington, 1953; Andres, 1953; Grobstein and Zwilling, 1953; Zwilling, 1954). Before taking up the specific application of these techniques to problems of cell stability, a brief account may be in order on the procedures themselves.

It is worth recalling that, though artificial tissue disaggregation is a tool for investigation, natural tissue disaggregation is of frequent occurrence in normal and abnormal development. Gametogenesis, hematopoiesis, blastema formation in regeneration, and metastasis or exfoliation in malignancy are all examples of tissue disaggregation in the sense used here. Whenever the characteristic architecture of a tissue is disrupted, by whatever means, with release in greater or lesser degree of viable component cells into the environment, disaggregation or dissociation may be said to have occurred. The processes involved in the release, as well as those involved in subsequent reaggregation or its failure, are of interest in understanding the mechanisms of tissue organization, as well as the relation of tissue properties to the properties of their component cells.

For these reasons, artificial disaggregation was attempted early in the history of experimental morphology, employing such direct mechanical procedures as cutting, grinding, shaking, and sieving. Tissue culture later provided a more physiological and subtle method of disaggregation when, in favorable media, explants tended to spread and disperse, though not always to the cellular level. The dissociative effect of ionically unbalanced solutions and of high pH have both been employed (Holtfreter, 1948) and, lately, enzymatic digestion alone or in combination with other methods has been used to break up tissue matrices and to liberate cells. Each of these methods has its advantages and disadvantages, and there is little evidence as to the equivalence of their effects. It may be noted, however, that the variety of older and newer methods now available has much increased the facility with which problems involving tissue dissociation can be approached.

Dissociation techniques have been used with various objectives in mind. Some investigators have studied the reconstituent process itself, *i.e.*, how far dissociation can be carried and still permit the organization of a new supracellular entity, what amounts of tissue are required, what events occur, what cellular interactions take place. Others have been interested primarily in obtaining cellular suspensions for inoculation into culture vessels or living hosts, or for quantitative studies of relatively homogeneous cellular populations. Still others, and the work to be described is in this category, have used dissociation to gain information on the degree of intrinsic type-stability of cells after disruption of their customary environment.

The rationale for this last procedure is as follows. With few exceptions, cells which lie quiescent in organized tissues tend to preserve their type. So long as they are undisturbed, it cannot be told whether their stability is due to intrinsic factors ("cytodetermination") or to their constant relation with other cells and the intercellular matrix. If the cells are freed completely from their original associations and then reaggregate, original types with intrinsic stability should reappear, whereas cell types whose stability depends upon factors extrinsic to the cell may not reappear. Unfortunately, this fairly clear-cut

principle is complicated in practice by a number of uncertainties, namely; whether the dissociation kills or impairs some cells; whether it is complete enough to free *all* cells from possibly stabilizing associations; whether the cells reaggregate and establish their original extrinsic relations so quickly that a false impression of internal cellular stability is gained.

In practice, if reaggregation occurs, the product may lack some cell types present in the original (simplification), may have the same cell types (reconstitution), or may show new cell types not previously present (regulation). Each of these cases has its own complexities of interpretation, and we shall concern ourselves for the moment only with the first. It seems safe to assert that cell types which fail to appear, following disaggregation, could not have been intrinsically stabilized in the original cell mass, provided that the procedure does not preclude recognition of the cell type in question, and that the total loss of cells during the procedure is not greater than the number present, or to be expected, of the type in question.

Several years ago, while studying the behavior of mouse embryonic shields in tissue culture and on transplantation to the eye, simplification of differentiative behavior was noted when early intact shields were cultured at a glass-clot interface prior to implantation into the eye (Grobstein, 1951). Nervous tissue, for example, was regularly found in the teratoid growths produced by direct implants, but was rarely present following several days of preculturing. At later stages (head process), intact shields, even though precultured, yielded implants with nervous tissue, but shields which had been cut into fragments (Grobstein, 1952) prior to culturing and all fragments explanted in a close cluster showed declining incidence of nervous tissue in the subsequent implants as the fragmentation was progressively increased from halves to one-sixteenths (FIGURE 1). At the one-sixteenth level, although the explant mass included the same amount of tissue as with the intact shields (except for the relatively small number of cells damaged in cutting), nervous tissue was virtually absent.

At still later medullary plate stages, fragmentation of the presumptive nervous tissue to pieces of approximately the same size failed to prevent the formation of nervous tissue in the reaggregated and implanted mass. The impression derived from these results was that the tendency to form nervous tissue is gradually rising in the embryonic shield during primitive streak stages, but that, even as late as the head process stage, the tendency can be disrupted by fragmentation and culturing of the tissue. The result emphasized that a part or region of the embryo which is sufficiently stable to continue on its presumptive course when isolated as a whole (the accepted test for "determination"), does not necessarily consist of cells which are intrinsically fully stable in their definitive type.

The mouse data are supported and amplified in this conclusion by results of similar experiments on the definitive primitive streak blastoderm of the chick (Grobstein and Zwilling, 1953). Because of the larger size of the chick blastoderm, and the greater background of knowledge of its behavior, the experiments could be carried out with small regions of the blastoderm of known prospective fate. The regions in question, C, $L_{1,2}$, $R_{1,2}$ (FIGURE 2), all of equal size, were cultured for five days at a glass-clot interface, as with the mouse

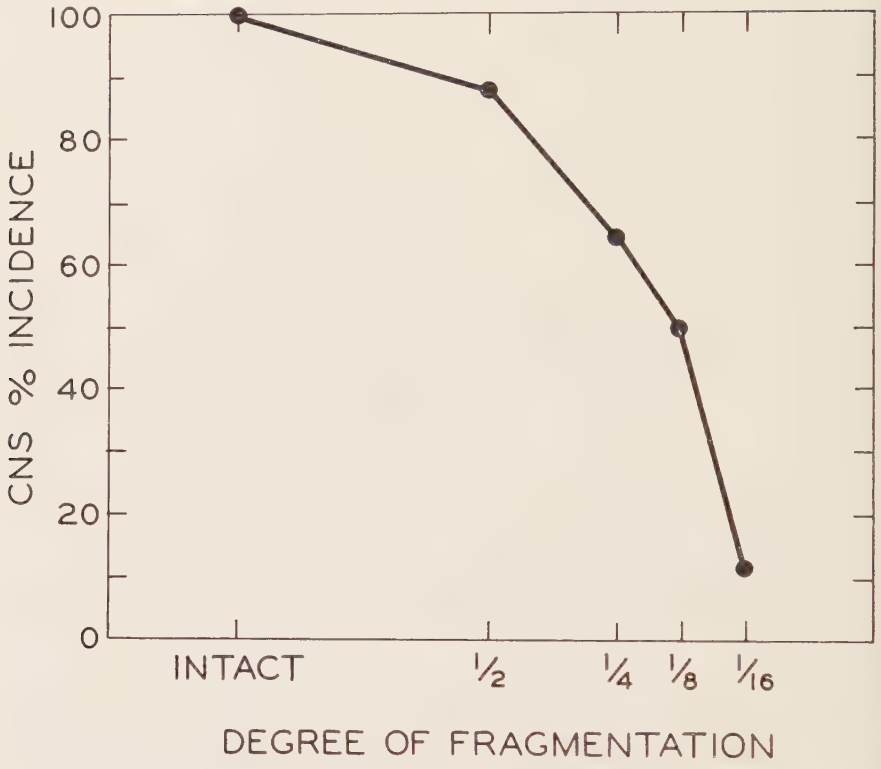

FIGURE 1. Effect on percentage incidence of central nervous tissue of progressive fragmentation of mouse embryonic shields. Level of fragmentation plotted in terms of relative area of final fragment.

material, and then transplanted to the chorio-allantoic membrane of chick embryos on the tenth day of incubation to continue their growth and differentiation. Each region was transplanted intact, both with and without prior culturing, and after culturing of a close cluster of fragments obtained by cutting the original piece into halves, quarters, eighths, or sixteenths. It will be noted in FIGURE 3 that prior culturing tended strongly to increase, rather than decrease, recoverability and weight on the chorio-allantoic membrane and that, considering all cases collectively in which fragmentation was not carried beyond eighths, the incidence of nervous tissue was actually increased by culturing. To be noted, too, is the fact that the No. 1 piece is well within a "determined" neural area, since pieces both central and peripheral to it also give neural tissue on direct grafting to the chorio-allantoic membrane.

FIGURE 4 makes clear, however, that when fragmentation is carried one cut beyond one-eighth, the graft weight and incidence of nervous tissue drop off sharply. This result on a *small part* of the chick blastoderm repeats that obtained with the *whole* mouse embryonic shield, and indicates that what is involved is not the fractional value of the piece with reference to a whole but the absolute size of the fragment. In fact, the fragment size in the two cases,

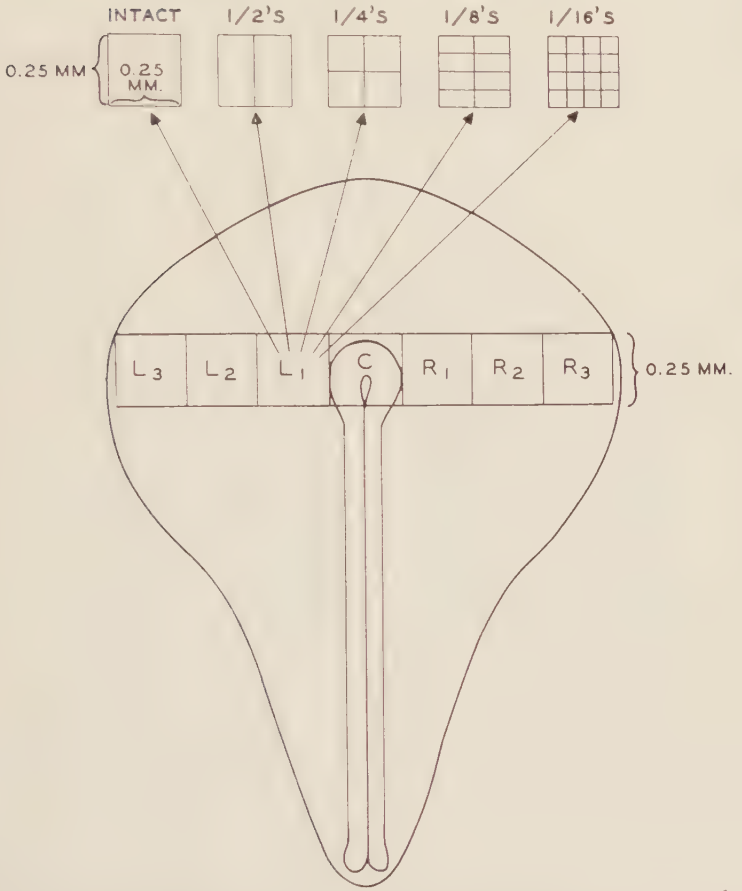

FIGURE 2. Diagram of definitive primitive streak chick blastoderm showing sources of tissue and manner of fragmentation.

though somewhat variable, is of the same order of magnitude, *i.e.*, something less than 0.1 mm.

Further data on the chick, however, indicate that fragment size is not the only significant factor, but that what may be called "effective explant mass" is also involved. Concentrating on No. 1 pieces cut into eighths or sixteenths and, varying the number of fragments and their dispersion in the original explant, results were obtained which are summarized in TABLE 1. Where the explant included more tissue than contained in a one-eighth fragment (*i.e.*, more than one-eighth or two-sixteenths), and all fragments were in a close cluster, the effect of fragment size is shown in the 88.6 per cent and 29.6 per cent incidences of nervous tissue for one-eighth and one-sixteenth fragmentation respectively. Similarly, where the amount of tissue is that in a one-eighth fragment (one-eighth or two-sixteenths) the difference between 42.9 per cent and 0 shows the effect of fragmentation to one-sixteenth size. What is inter-

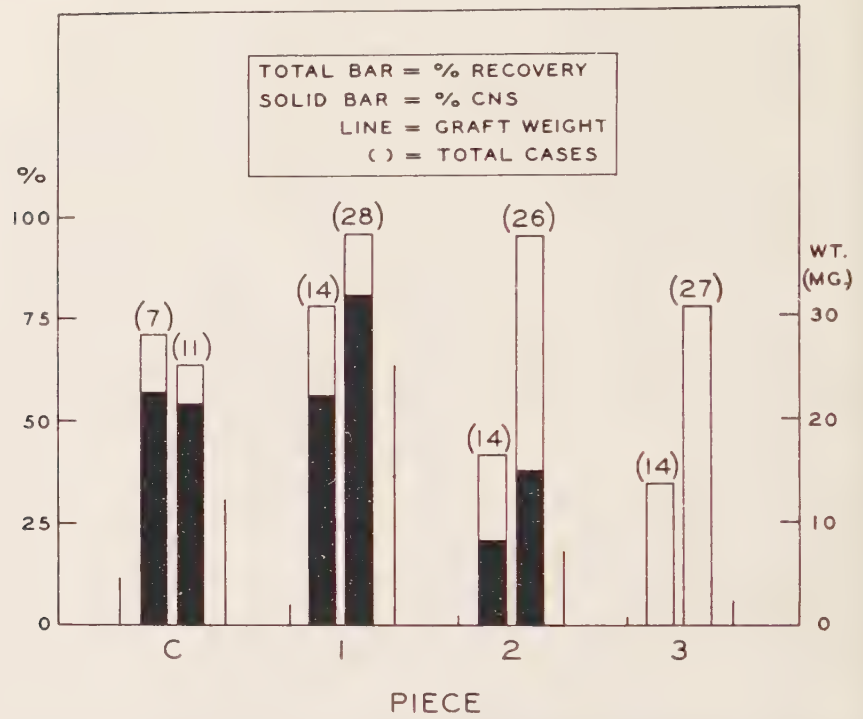

FIGURE 3. Behavior of the several chick blastoderm pieces grafted to the chorio-allantoic membrane with and without previous culturing. For each type of piece, the left line and bar represents noncultured, the right line and bar represents cultured piece behavior. Data here include only fragmentation down to one-eighth. There is no unfavorable effect of culturing on incidence of central nervous tissue (black bars), and the No. 1 piece clearly is within the presumptive neural area.

esting, however, is that when one-eighth fragments were explanted in dispersed clusters to force them to spread before re-fusing into a single mass, even as many as eight fragments (equivalent in mass to an intact piece) show little higher incidence of nervous tissue than the single one-eighth fragments. The incidence is significantly lower than in the cases where like numbers of one-eighth fragments are placed in close cluster where they can fuse rapidly. Similarly, dispersed clusters of one-sixteenth fragments produce no nervous tissue in contrast to the 29.6 per cent incidence in close clusters.

These observations suggest that, though there may be a limiting value below which fragmentation cannot be carried without eliminating neural differentiative tendencies, there is also a critical mass or, possibly, cellular density, which must be achieved at some subsequent time if neural tissue is to appear. Under the conditions of these experiments, isolated one-eighth fragments (of variable absolute size because of limitations in the precision of cutting) achieve this critical mass in about half the cases, and nearly always do when two or more are placed in close clusters. One-sixteenth fragments, on the other hand, never achieve the critical mass when isolated but, occasionally, can do so when combined in close clusters. The whole body of data, on chick and mouse, is

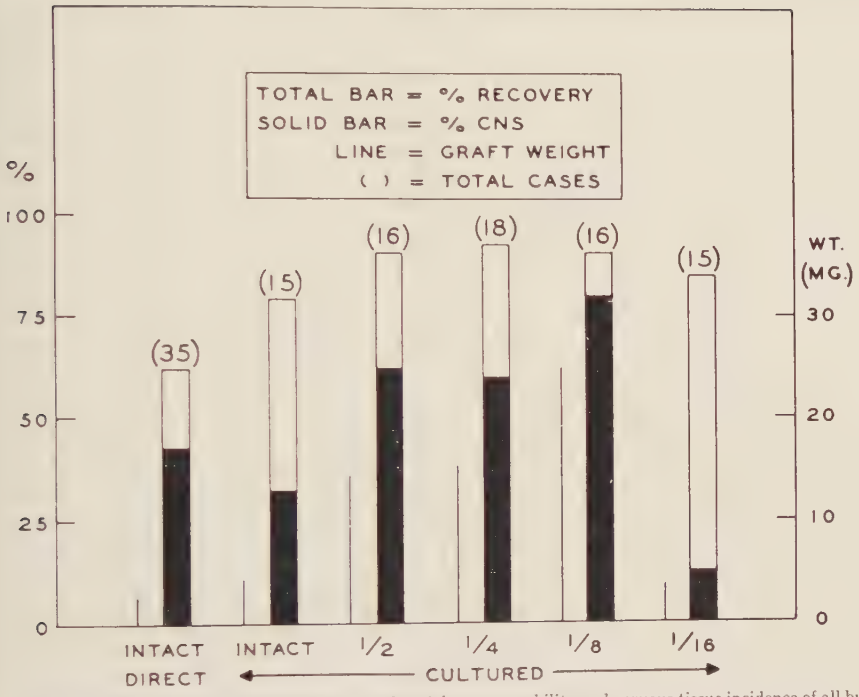

FIGURE 4. Effect of prior fragmentation on graft weight, recoverability and nervous tissue incidence of all but No. 3 pieces on grafting to the chorio-allantoic membrane after culturing

TABLE 1

EFFECT OF CLOSE VERSUS DISPERSED CULTURE ARRANGEMENT OF NO. 1 FRAGMENTS ON GRAFT BEHAVIOR

Arrangement in culture	1/8 Fragments			1/16 Fragments		
	Viable hosts	Weight	% CNS incidence	Viable hosts	Weight	% CNS incidence
		mg			*mg.*	
Close clusters mass greater than 1/8	35	19.3	88.6	27	3.4	29.6
Mass = 1/8	7	7.0	42.9	7	0.01	0.0
Dispersed clusters or mass less than 1/8	12	6.3	50.0	28	0.24	0.0

in conformity with the idea that explants placed at a glass-clot interface are simplified in their subsequent differentiative behavior when the spreading and disaggregative behavior induced at this site prevents achievement of a critical aggregate mass, within which processes essential to neural formation can occur. Initial level of fragmentation may be a direct factor, or it may be involved only in that small pieces are more quickly and more completely spread. Information on this latter point might be gained from tests of fragments, at a one-sixteenth level or below, which are allowed to reaggregate prior to explantation.

The observations also suggest that, at the stages tested in mouse and chick, when nervous tissue "self-differentiates" in intact pieces, the cells themselves have not yet acquired fixity of type as nerve cells. This is to say that, in this particular instance of determination of new properties in the embryo, some stabilization at the tissue level seems to precede stabilization at the cell level. Before this conclusion is accepted, attention should be given to alternative explanations; *i.e.*, that determined nerve cells were present, but somehow eliminated during the procedure; that they were present but not recognized; or that their absence is an artifact of culture and of no significance for normal processes.

None of these explanations seems satisfactory. It is not easy to see how the last cut, randomly made, which converted one-eighth fragments to one-sixteenth fragments (particularly in the chick material where the No. 1 piece would be expected to be fairly homogeneous) could have specifically eliminated virtually all determined nerve cells. Nor does the difference in behavior of close versus dispersed clusters lend itself to explanation in these terms. Certainly, the number of cells initially destroyed in cutting would be the same in close versus dispersed clusters, and there was no evidence for differing degrees of necrosis in culture. In fact, in none of the experiments was there evidence of significant necrosis, certainly not enough to account for the elimination of specific cell types. Nevertheless, the formal possibility must be conceded, until more quantitative methods are employed or specific cells followed, that determined nerve cells may have been lost.

The possibility that nerve cells were present but unrecognized may be regarded as exceedingly small, particularly in the mouse material, where the level of differentiation of implanted tissues was equivalent to that in the adult. Identification in all cases was based, not on the cultured material, but on subsequent transplant behavior. Nervous tissue, when present, was readily recognizable, and there were very few instances where other tissues were not easily classified as specific types. Moreover, nervous tissue was nearly always in conspicuous, well-organized masses, and even scattered ganglion cells were easily identifiable.

Nor does it seem likely that we are dealing with a culture artifact, *i.e.*, an antidifferentiative effect based on nutritional inadequacy or similar factors. Explanted embryonic central nervous tissue survives quite well under the conditions used, and makes its appearance readily in explanted medullary plate fragments of the same size. Moreover, under these same conditions, morphogenesis of embryonic rudiments continues, induction can occur, and new cell types can arise (Grobstein, 1955). There is no reason, therefore, to regard the simplification effect, as indicated by the failure of appearance of nervous tissue, as a culture artifact rather than a developmentally significant phenomenon.

Perhaps the best evidence, however, that the simplification is real and significant lies in reports of the widespread occurrence of related phenomena in other situations. From protozoa to vertebrates (Berrill, 1945; Holtfreter, 1951), there appears to be a limiting minimum mass essential to continued morphogenesis and differentiation. From the minimum mass to an optimal

one, increasing complexity of organization becomes possible and subsequent differentiation is increasingly varied. Following dissociation, for example, sponges do not reconstitute from aggregates smaller than 0.1 to 0.5 mm. in diameter (Brien, 1937), and similar figures are given for the hydroid, *Corymorpha* (Child, 1928). These dimensions are very close to those of the smallest fragments which would yield nervous tissue in the mouse and chick experiments, and it is of some interest that Papenfuss (1934) found that aggregates of *Hydra* fragments of approximately this size would yield new individuals, though single fragments reportedly would not (Peebles, 1897). The similarity with the close and dispersed clusters in the chick experiments reported above is striking.

Related effects are reported in vertebrates. Weiss and Amprino (1940) noted a minimum size below which explants of prescleral mesenchyme of the chick would fail to produce cartilage in culture. Particularly striking and relevant to the present discussion are the results of Lopaschov (1935), who isolated in salt solution fragments of head mesenchyme from amphibian gastrulae. Where explants were made up of only one or two such fragments, only striated muscle differentiated but, on increasing the mass by fusing increasing numbers of fragments, the size and complexity of differentiation increased. With 6 to 10 fragments, in addition to muscle, there resulted chorda, epidermis, brain, and eye with associated lens and pigmented epithelium, and the whole assemblage of tissues was in a definite if not normal organization. Lopaschov noted that, since all levels from head to tail appeared to be represented, the larger aggregates composed of original head mesenchyme must have undergone a new axiation. It is noteworthy that the nervous tissue was produced by head mesenchyme, not ordinarily thought of as containing precursor cells of central nervous type.

Something similar appears to be involved in the results recently reported by Andres (1953) who described teratomas produced in the chick by intravenous injection of cellular suspensions of dissociated embryos or portions of them. Andres found a correlation between size of the teratoma and its complexity of differentiation, noting particularly that nervous tissue appeared only in the largest of the growths, and then only in association with several other tissues. Andres is inclined to interpret his results in terms of critical minimum amounts of material required for each tissue type.

Against this background, it seems justifiable to interpret the mouse and chick experiments as indicating that tissues which can "self-differentiate" nervous tissue on isolation from the rest of the embryo, the classical test for "determination," need not be composed of cells of fixed or determined neural type. It follows that, in the appearance and stabilization of neural tendencies in these cases, fixity of cell type does not occur suddenly at the beginning of the process. It is of interest, in this connection, that King and Briggs (1954), with their ingenious technique for nuclear transplantation in amphibia, have found no indication of nuclear "differentiation" in late gastrula stages, regarded as "determined" for nervous tissue by usual criteria. In a process disrupted by spreading or dispersion of the cellular mass, and with no evidence of intrinsic cellular stability, the question may be considered as to whether the beginnings

of neuralization do not lie in an altered organization of the mass, where this decried (*vide* Bonner, 1952) but essential word refers to the totality of interrelations among the cells and the intercellular matrix.

This question is given point by at least two other observations on developing systems *in vitro*. It has been shown (Grobstein, 1953) that the submandibular salivary rudiment of the mouse develops, fairly early, an apicobasal regional character such that the terminal bulb on explantation will undergo dichotomous branching, whereas the explanted basal stalk remains quiescent and forms a cyst. The branching behavior leads to the formation of terminal alveoli, the cells of which become the characteristic secretory cells of the submandibular gland. Cells not involved in the alveoli become simple duct epithelium.

By the test of explantation, the bulb would usually be thought of as determined to form at least some secretory tissue, and the basal stalk to form only duct. Precursor cells of secretory type might be expected to be present in the bulb but not in the stalk. In fact, the branching and subsequent production of secretory cells by the bulb epithelium is dependent on association with its surrounding mesenchyme and is completely inhibited by separation of the two components. Conversely, while the intact stalk undergoes no dichotomous branching leading to the formation of alveoli, stalks cut into small fragments and explanted in cluster with pieces of capsular mesenchyme of the bulb region show slow weak branching of many of the fragments to form terminal alveoli (*ibid.*). Although such cultures have not been tested by reimplantation, there is every reason to expect, from other experience, that they would yield secretory type cells. The impression, therefore, is strong that the establishment of a differential between apex and base in the submandibular rudiment, despite its histogenetic consequences, represents, not a determination of new cell types, but some system-dependent change, reversible upon disturbance or disruption of the system.

The same impression arises from still incomplete data on the *in vitro* induction of kidney tubules by embryonic spinal cord in metanephric mesenchyme (Grobstein, 1955). Such mesenchyme, isolated with trypsin from the metanephric rudiment of 11-day embryos, never produces tubules when cultured alone. When fragments are clustered around a piece of 11-day dorsal spinal cord, however, tubule rudiments appear in close association with the periphery of the cord. The same effect occurs when spinal cord and mesenchyme are cultured on opposite sides of a membrane filter. This procedure allows the interacting tissues to be reseparated at any point in the inductive process. Mesenchyme thus exposed to spinal cord for 24 hours and then cultured separately fails to form tubules, but mesenchyme exposed for 48 hours does continue tubule-formation on subsequent isolation. The significant point is that, between 24 and 48 hours, morphologically recognizable tubule rudiments are appearing. It seems from presently available data that tubules, with their new epithelial cell type, do not continue to form unless they have well begun before separation. Once again, the initial event in the rise of a new cell type appears not to be cellular fixity, but an alteration of tissue organization which itself takes time to stabilize before the cellular change is insured.

The general conclusion that seems to emerge from the data which have

been presented is that stabilization of properties in developing tissue masses, or what has been called determination, tested for by simple isolation of the mass from its surroundings in the embryo, is not necessarily accompanied by stable system-independent alteration of cell type, *i.e.* cytodifferentiation as defined by current usage. Studies of stabilization of tissue type, upon which depend most of our knowledge and concepts of determination and differentiation, therefore tell us little about this kind of cytodifferentiation. This point is not new, but it seems important to reemphasize it here when our attention is directed toward "molecular events" in cellular differentiation. Stabilization of properties at the tissue level *may* involve intrinsic cellular change, but it may equally well involve change in cell relations (grouping, layering, folding) or change in the intercellular matrix, or both.

It follows that, if we wish to correlate, with precision, molecular events with the time and progression of type change in *cells*, data on the behavior of *intact tissues* cannot be relied on. Such data, for example, have given rise to the conception of "chemodifferentiation," referring to the period when a tissue has acquired a preferred tendency revealed by transplantation or explantation, but not correlated with any recognizable morphological change in the cells. The assumption is that the new tendency is due to a *determinative* intracellular molecular alteration, that a new cell type has already arisen, which requires only time and favorable circumstance to manifest itself. The assumption may be correct in particular instances, as recently stated for amphibian notochord cells by Mookerjee, Deuchar, and Waddington (1953), but it can be made only on the basis of study of the behavior of dissociated tissues to exclude the possibility that what is interpreted as cellular stabilization of type is not, in fact, tissue stabilization. To extrapolate from the latter to the former, and then to seek correlation with molecular events *within* the cell, can obviously lead to confusion.

It would appear, therefore, that in discussing the molecular events in cellular differentiation, as we are doing, with attention focused on molecular specificities, metabolic pathways, and substrates in nucleus and cytoplasm, we had best keep in mind the old-fashioned but nevertheless largely unsolved problems of supracellular organization. In the relations of cell to cell, and of cell to matrix, may lie at least part of the explanation of the rise and stabilization of new properties in development.

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Discussion of the Paper

Doctor H. Clark Dalton (*New York University, New York, N. Y.*): Do you think that much of the confusion in our discussion of our basic concepts of determination and differentiation stems from the fact that we are still suffering from mental limitations imposed by the Cell Doctrine?

Doctor Grobstein: Doctor Dalton's question is both provocative and pertinent. The discovery of the nearly universal cellularity of living things was a revolutionary advance which underlies most of modern analytical biology. The rapidly expanding knowledge of cells is, of course, in no sense mentally limiting. Rather, it has opened up new avenues toward generalized biological theories. But Doctor Dalton has referred not to our knowledge of cells, but to the Cell Doctrine, which I shall take to mean, in brief, the notion that the cell is so central and primary in biological phenomena that from its properties can be extrapolated at least the larger part of what we know or care to know about the properties of living things. This notion has indeed contributed to our conceptual difficulties about determination and differentiation because, operating in reverse, it has led us to pack into the cell a multitude of naked hypothetical "determiners" to "explain" the subtle properties of highly complex systems.

The question invites a discussion of methodological problems that would go far beyond our immediate purpose. I should like to make one point quickly, however, at the risk of oversimplification. Above the Protista, all living organisms are cellular systems at one or more levels. We are all aware that the properties of the organism cannot be arrived at by any simple summation of the properties of the cells, and we tend to explain what is more or less than the sum in terms of relations existing among the cells which alter their properties when in the system. But an additional important and very real part of the system may thus be overlooked—the intercellular matrix. All systems, mechanical, electronic or biological, consist of components and intercomponental space. In physical systems, the intercomponental space usually is a relatively insignificant factor in contributing to the *specific* properties of the system. In biological systems, we should be aware, thanks to such concepts as internal *milieu* and homeostasis, that the intercomponental space is of far

greater significance, both as a system stabilizer and as a *determiner* of specific system properties. In fact, many biological systems owe their beginnings to the establishment of a boundary; *i.e.*, the appearance of a matrix to some degree different from the general environment. Part of the conceptual difficulty referred to by Doctor Dalton may lie in our overemphasis on the cell at the expense of the matrix in explaining change and stabilization of change in cellular systems. The thought has the advantage of suggesting a target for experimental approach.

LIMB REGENERATION AND DIFFERENTIATION OF "OVERT BEHAVIOR" IN URODELES AS STUDIED BY MEANS OF THEIR RESPONSE TO CHEMICAL AGENTS

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There may be no adequate reason for grouping limb regeneration and "overt behavior" under the same heading, but the candid reason for it is that both these studies were pursued simultaneously. The following rationalization came as an afterthought. It appears to me that chemical agents that have affinities to specific developmental differentiations may serve as their markers. By varying the chemical structure of the agents and relating this variation to the change in their effect, an insight can be gained into the chemical nature of the differentiations. The prerequisite for this type of study, however, is that the response to these agents must be easily quantitated or, at least, it must be plastic. An all-or-none response would be less convenient. I shall try to show in this paper that such a graded response to chemical agents obtains both in the case of regeneration and of "overt behavior." It can be suggested that this wide range of responsiveness can be obtained more easily at a more advanced organizational level than in the early embryo. Thus, the common denominator in the study of regeneration and "overt behavior" by means of their reactions to chemical agents is their wide range of reactivity to these agents. In the case of either of these two phenomena, it is the purpose of this paper to present preliminary attempts to demonstrate steps in differentiation which may not be demonstrable by other means. Indeed, in the case of the "overt behavior," Weiss (1939) suggested that its development may depend on changes undetectable by the current embryological and related methods.

"Overt Reactions" of Urodele Larvae to Drugs

About six years ago, Doctor Koppanyi and myself were testing the responses of larval urodeles to several stimulants of the central nervous system in the hope of using them as an assay medium for convulsants. As can be seen from TABLE 1, larvae reacted by appropriate convulsions to such common central nervous system stimulants as strychnine, metrazol, and anticholinesterases. We were somewhat surprised, however, to notice that coramine failed to produce convulsions since we were accepting, at that time, the notion that coramine was a spinal and medullary convulsant (Koll, 1937). Our observation, however, made us go into the literature more deeply to discover that, more recently, there had arisen a controversy as to the actual site of the action of coramine within the central nervous system, and that the cerebral cortex or psychic areas cannot be as yet excluded (Maloney *et al.*, 1932; Hahn, 1941; Ten Cate *et al.*, 1945). Most of the conclusions of the studies quoted are based on ablation experiments which are open to certain criticisms. It may be that a larval salamander is Nature's own ablation experiment. Then, the absence of coramine convulsions in urodele larvae would be due to the absence, in these

TABLE 1

EFFECTS OF CENTRAL NERVOUS SYSTEM STIMULANTS AND RELATED AGENTS ON THE "OVERT BEHAVIOR" OF AMBLYSTOMA PUNCTATUM, STAGE 60 (GLÜCKSOHN) LARVAE. MOLAR CONCENTRATIONS

Drug	Number of animals	Concentrations		CNS Effect	Remarks
		Minimum effective	Lethal		
DFP	80	10^{-9}	10^{-5}	1. "Tail Twitch" 2. Myoclonic convulsions 3. Tonic convulsions 4. Paralysis	Latent period depending on concentration
Prostigmane methyl sulfate	40	2×10^{-7}	10^{-5}	Myoclonic and tonic convulsions. Paralysis.	Latent period depending on concentration
TEPP	12	?	10^{-7}	Myoclonic and tonic convulsions. Paralysis.	
Nicotine Salicylate	40	3×10^{-7}	10^{-6}	Inconclusive hyperexcitability with concentration 10^{-7}. No convulsions. Paralysis with 3×10^{-7} solution.	One day latent period with 3×10^{-7} solution. Some recovery on transfer into Holtfreter.
Atropine sulfate	35	?	3×10^{-7}	?	No typical convulsions. Fatal period depends on concentration.
Strychnine sulfate	30	5×10^{-19}	5×10^{-5}	Myoclonic and tonic convulsions	Irreversible paralysis after one month (opisthotonus)
Metrazol	40	10^{-4}	10^{-4}	Myoclonic and weak tonic convulsions	Latent period of one day. Some reversibility of the CNS effect.
Nikethamide	35	None	5×10^{-5}	No convulsions observed	Fatigue and paralysis with lethal concentrations
Strychnine } Atropine }	30	$10^{-9} \times 10^{-9}$	?	Myoclonic and tonic convulsions. Paralysis within 72 hours.	Negligible latent period.

TABLE 2

MINIMUM EFFECTIVE ANESTHETIC CONCENTRATIONS FOR AMBLYSTOMA PUNCTATUM LARVAE

No. of animals	Larval length (mm.)	Ethyl alcohol	Acetanilid*	Chloral hydrate	Chlorobutanol	Sodium barbital	"MS-222"	Nembutal	Paraldehyde
8	10–12	1:33	1:1000	1:250	1:2000	1:20	1:7500	1:500	1:200
10	13–16	1:33	1:1000	1:500	1:4000	1:25	1:10,000	1:1000	1:200
12	17–19	1:40	1:1000		1:4000	1:25	1:10,000		1:400
30	20–22	1:40					1:10,000		
20	23–27								
15	28–33	1:40							
15	34–40	1:50	1:1000	1:500	1:10,000	1:50	1:10,000	1:2000	1:400
15	41–45				1:15,000		1:15,000		

* Concentrations of acetanilid *1:2000* and *1:1500* have been found ineffective throughout the development.

forms, of the appropriate site for the action of coramine. This suggestion would indicate, in turn, that the coramine effect requires the presence of the higher centers (Karczmar and Koppanyi, 1947). This led us to the realization that the "overt reaction" to an agent could serve as a marker of the presence, in the organism, of an appropriate site of action and that our observation concerning coramine bears some resemblance to the correlation established by Coghill (1929) between the "overt behavior", *i.e.*, the progressive appearance and development of the early movements, swimming, feeding and locomotion, and the morphogenesis and histogenesis of the nerve and muscle systems. Thereupon, we proceeded with an attempt to establish whether "overt reactions" to selected agents acting on specific organ systems of larval urodeles change progressively during the development. When this question could be answered in the positive, it was hoped to relate these reactions to anatomical or biochemical developmental differentiations.

The reactions were accordingly studied on *Amblystoma punctatum* and occasionally on *A. opacum* larvae from the first appearance of motility (Harrison's Stage 34) until premetamorphic stages were reached (Glücksohn, 1931, Stage 60). Three classes of substances were used: central nervous system depressants, anticholinesterases, and curarimimetic agents.*

In the case of the central nervous system depressants, the surgical anesthesia tested by appropriate stimuli was the "overt reaction" studied. Two criteria, the minimal effective anesthetic concentration and the length of the induction period of anesthesia, could be employed to test whether this reaction changed during the developmental period specified. It was soon noticed that there was a change in both these quantities (FIGURES 1 and 2, TABLE 2), at least in the case of some agents. This change could be quite drastic and occur abruptly at some developmental period in the case of chloretone, chloral hydrate, and the barbiturates. Moreover, the change may have occurred at different developmental periods (compare chloral hydrate and the barbiturates, *cf.* FIGURE 1). Conjectures and further experimentation were now indicated. It had to be first ruled out, however, whether a change in the absorption in the course of

* For the techniques employed see Karczmar and Koppanyi, 1948a, 1953; Koppanyi and Karczmar, 1948; and Blum, unpublished.

development could not account for the results observed. Some pertinent experiments have been already described (Karczmar and Koppanyi, 1948b) which seemed to rule out this possibility. Another experiment, not described previously, was to immerse a group of young larvae, Harrison Stage 45, in the minimal effective concentration of chloretone until anesthesia ensued and then repeat the process with similar groups of larvae of the same stage until the solution ceased to be effective. This experiment was then reiterated with older Stage 60 (Glücksohn, 1931) larvae. The number of immersions necessary to render the solution ineffective was much greater for the younger smaller larvae than for the older and heavier larvae, but the total weight of the larvae of either stage necessary to exhaust the solutions was found almost constant (FIGURE 3). This result can be explained in one way only: the absorption coefficient must be the same for the larvae of these two stages. Indeed, by assuming this result, one can calculate the number of immersions necessary to render the two solutions ineffective by the larvae of the two stages, and the

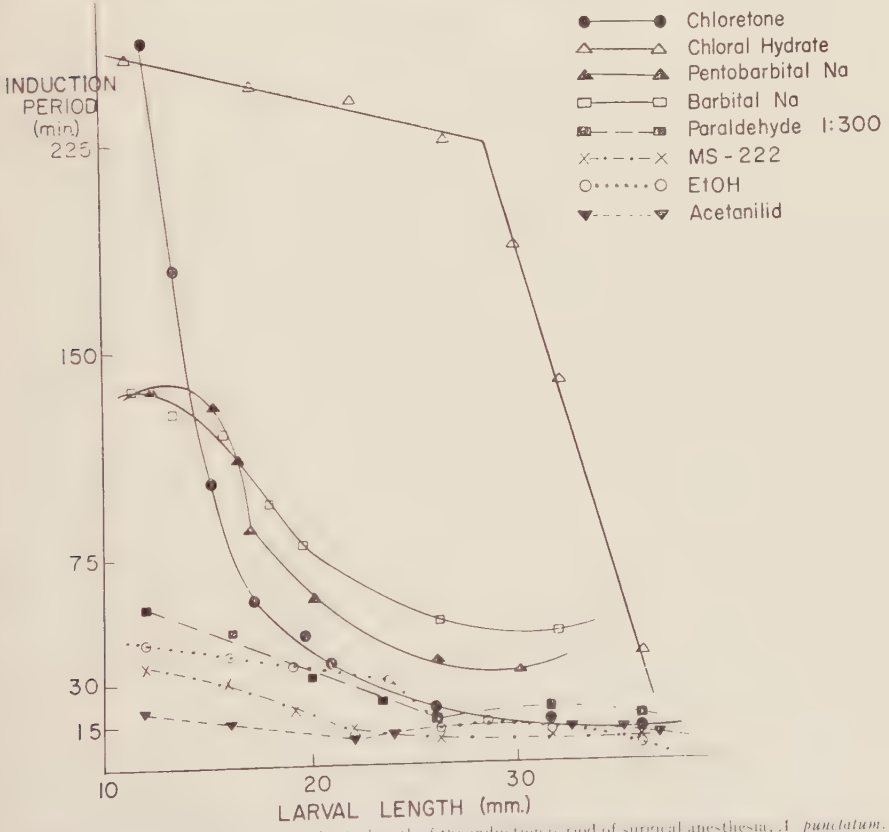

FIGURE 1. Developmental change in the length of the induction period of surgical anesthesia, in *punctatum*. Ordinate: induction period, minutes; abscissa: larval length in mm., larval length of 12 mm. corresponding to Stage 34-35 (Harrison). Throughout the development one constant (effective) concentration of each drug was used to induce anesthesia.

result of such a calculation agrees with the data of FIGURE 3 (*cf.* also Clowes *et al.*, 1937). More recently, we have been able to determine spectrophotometrically the amount of pentobarbital sodium absorbed at various developmental stages from solutions of equal concentrations, and we came out with essentially a similar result; namely, that the amount of the drug absorbed at different larval stages remains constant (Blum, unpublished data).

Thus, the effectiveness of the drug, rather than its absorption, changes with development, at least in the case of barbiturates and chloretone. It is now in order to seek mechanisms by means of which the development brings about this sensitization. It can be shown—accepting certain assumptions—that, if the relative number of the nerve cells increased in the course of development, this increase could constitute the mechanism desired. This explanation would have also the advantage of its great simplicity. The evidence for such an increase, however, is ambiguous. Koch *et al.* (1913) showed tachyauxesis for

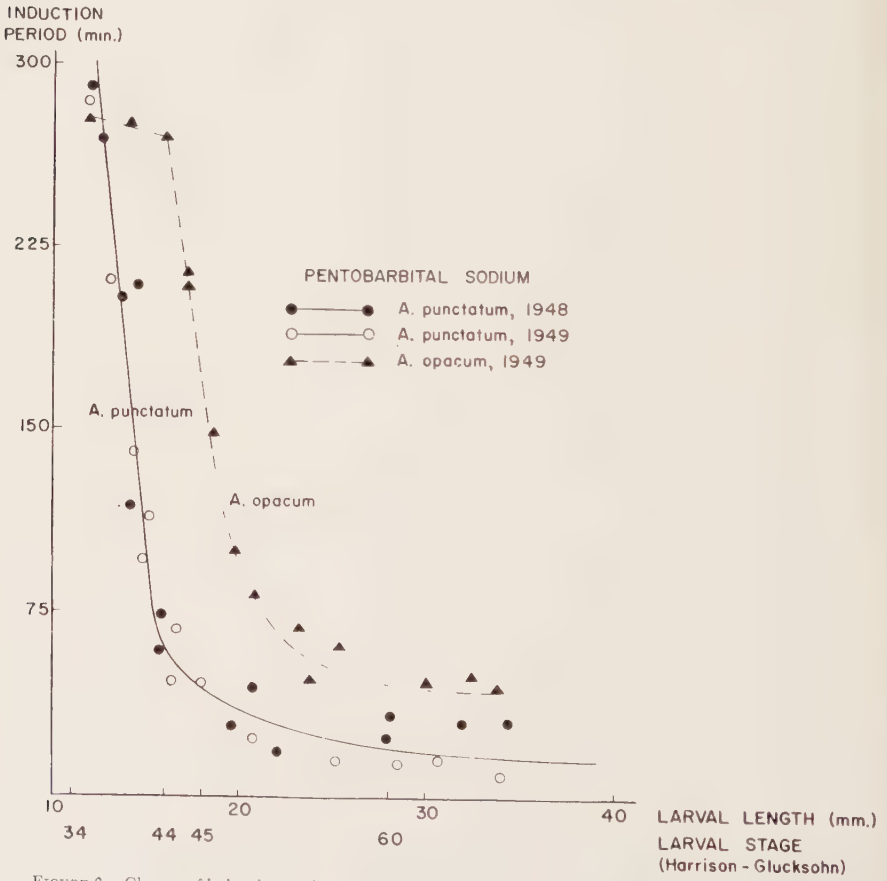

FIGURE 2. Change of induction period of pentobarbital sodium anesthesia throughout development. Measurements taken during two successive summers in the case of *A. punctatum* and during one summer in the case of *A. opacum*. For other explanations, see FIGURE 1.

phosphatides and cerebrosides of the rat brain, while the data of Scammon (1942) indicate a decrease of the ratio between the nervous and other tissue in the developing human. In the pertinent form, *i.e.*, salamander larva, the medulla at least grows somewhat less slowly than the body (FIGURE 4; *cf.* also Detwiler, 1945).

Of course, the rapid growth of one organ or even of a group of proteins relative to that of the rest of the body, is not as relevant as the differential growth of an enzyme determined as the basis of a particular "overt behavior" reaction. Before we return to the further analysis of changes in "overt reactions" to central nervous system depressants, I should like to present some data obtained from the study of agents affecting one such enzyme, acetocholinesterase, largely accepted as playing an important role in the processes of synaptic transmission. One of the agents used was diisopropyl fluorophosphate, DFP, an anticholinesterase affecting both the pseudocholinesterase and the acetocholinesterase (Augustinsson, 1950). Cholinesterase and, especially, acetocholinesterase have been related in higher forms to the development of motor "overt behavior" (Orr and Windle, 1934; Nachmansohn, 1940a and 1940b; Barron, 1941; Youngstrom, 1938). In salamander larvae, cholinesterase appears and begins increasing rapidly from the time of the appearance of the first movement (Coghill's "coil movement," Harrison Stage 34+). Thereupon, the enzyme concentration increases steadily until the beginning of the feeding (Sawyer, 1943a, 1943b). Moreover, the enzyme seems localized in the pertinent structures, the nerve and the muscle (Sawyer, 1943b). Finally, Sawyer observed (*op. cit.*) that the larvae exhibiting cholinesterase activity react to the anticholinesterase agent, physostigmine, while we observed that, in the case of DFP, the first "coil movement" of the Stage 34+ larva assumes a tetanic character. It is at this point, however, that difficulties appear. As

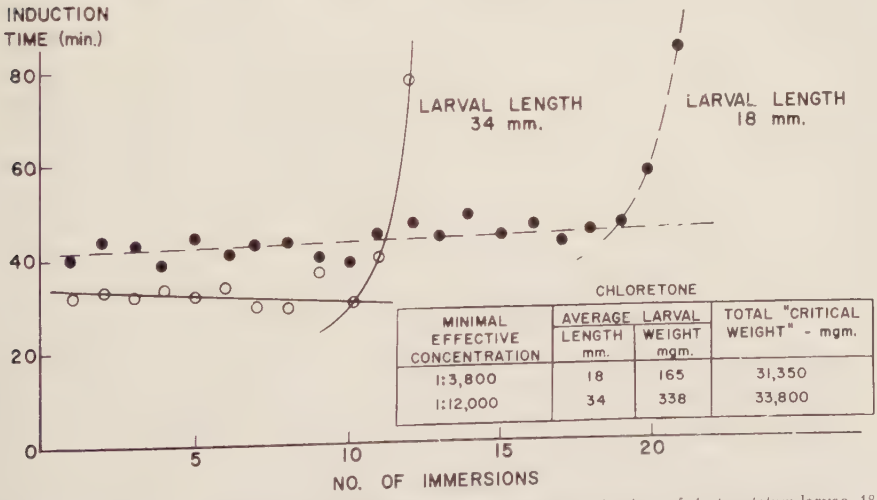

FIGURE 3. Loss of activity of chloretone solutions on repeated anesthetizations of *A. punctatum* larvae, 18 (Harrison Stage 45) and 34 mm. (Glucksohn Stage 60) long. Ordinate: induction time, minutes; abscissa: number of immersions. See also text.

FIGURE 5 indicates, the latency period of the DFP-induced tetanus depends on the DFP concentration and the speed of the induction of the tetanus continues increasing long after concentrations of DFP are reached which completely inhibit the enzyme in the larva (10^{-7} M; Karczmar and Koppanyi, 1953). There are further difficulties in relating the DFP effects on the larvae to cholinesterase: (1) the toxicity of this agent seems also to depend on its concentration rather than on the enzyme inhibition (FIGURE 5); (2) DFP is toxic to a similar degree to cholinesterase-containing and earlier, cholinesterase-free larvae (Karczmar and Koppanyi, 1953); (3) finally, functional recovery of the DFP-inized larvae occurs earlier than the reappearance of the enzyme activity (FIGURE 6). It is also of interest that the DFP dose-effect relationship changes its shape from Stage 34+ to 58 (FIGURE 7), although there is no change in the enzyme inhibition.

Thus, even if we try to correlate the larval "overt reactions" to an enzyme inhibitor, with the enzyme which is postulated to be the basis of these particular "overt reactions," and even though this enzyme shows the prerequisite tachyauxesis, the difficulties still remain present. This may be because we pay too much attention to the enzyme data without sufficient consideration of the effector system. In this respect, it is interesting to compare the "overt reactions" to DFP with those of another agent whose acetocholinesterase inhibitory activity may be secondary to its activity at the myoneural junction. This agent, WIN 8077 [N-N′ bis(2-diethylaminoethyl)oxamide bis-2-chlorobenzyl hydrochloride] (Karczmar and Howard, 1955), causes, at toxic levels, a curariform effect in higher forms (*op. cit.*) and also in larvae (Karczmar, unpublished data). This effect appears at the state of the probable appearance of the

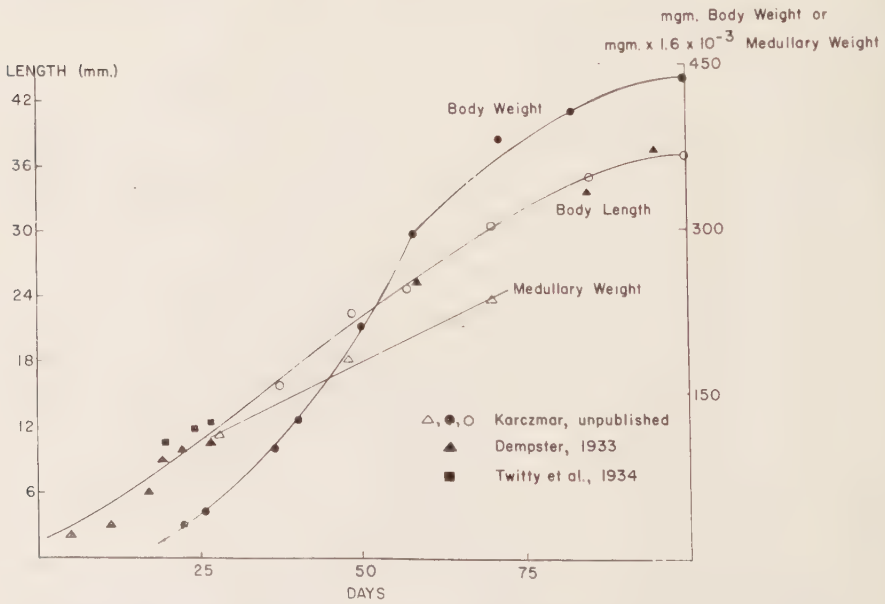

FIGURE 4. Growth of body length and weight and of medullary weight of *A. punctatum* larvae. Ordinate: larval length (mm.) and body and medullary weight (mgm). Legends in the figure self-explanatory.

functional myoneural junction (Coghill, 1929). The relationship between the concentration of WIN 8077 and the speed of induction of curariform effect appears to be that of an almost all-or-none phenomenon (FIGURE 7). Interestingly enough, the effect of curare seems to follow a similar relationship but what is even more striking, with a delay with reference to that of WIN 8077. In other words, curare is not yet active at the stage of the first "coil," *i.e.*, of the functionability of the muscle system.

In recent years, it proved particularly fruitful to predict the physiological action of chemical agents on the basis of their molecular structure and length, ionic charge, *etc.*, *i.e.*, on the basis of their chemical affinity to the effector (Nachod and Lands, 1953; Pelikan *et al.*, 1954). By virtue of its cationic electron density and molecular length, WIN 8077 belongs to compounds that have a great affinity to ganglionic and myoneural synapses. The data presented in FIGURE 7, as well as discrepancies between enzymic and physiological effects of DFP (FIGURES 5 and 6), may suggest that we deal here with subhistological level of differentiation revealed by its changing reactivity to pertinent agents. Indeed, at one level of differentiation, the affinity to one specific chemical agent may be still absent, while that to another may have already developed. The developmental change in the dose-effect relationship of DFP (FIGURE 7) may be due to just such a change of affinity underlying the apparent lack of change in the enzyme inhibition by DFP. It seems from

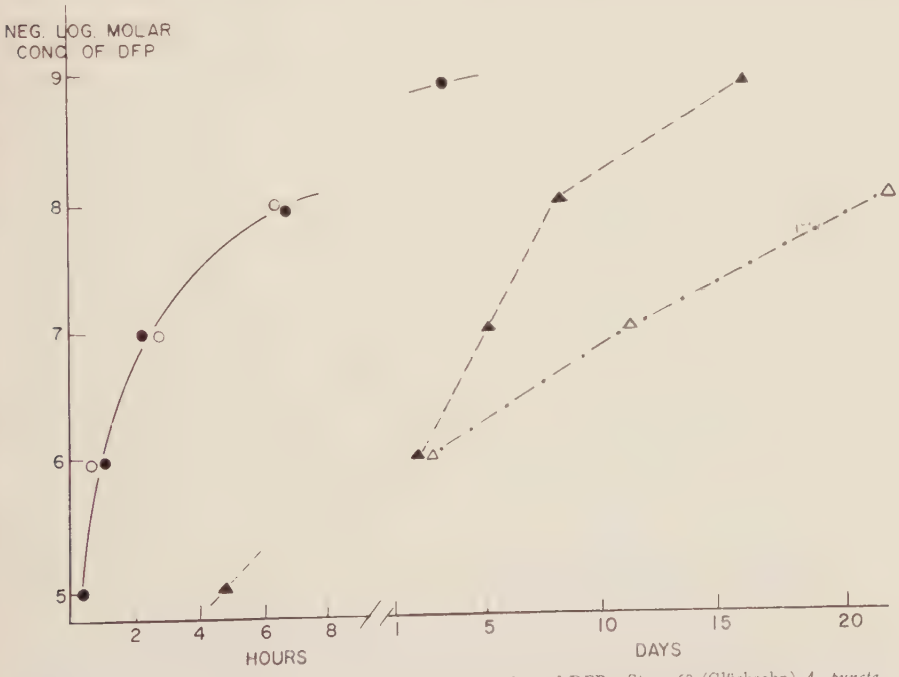

FIGURE 5. Latent period and toxicity of various concentrations of DFP. Stage 60 (Glücksohn) *A. punctatum* larvae. Full and open circles, continuous line: latent period of the "overt" effects of DFP alone and of DFP and atropine solutions, respectively. Full and open triangles: latent period of lethality of solutions, respectively, of DFP alone and of DFP and atropine. *Cf.* also Karczmar and Koppanyi, 1953. Ordinate: molar concentrations of DFP; abscissa: two scales—latent period in hours and days.

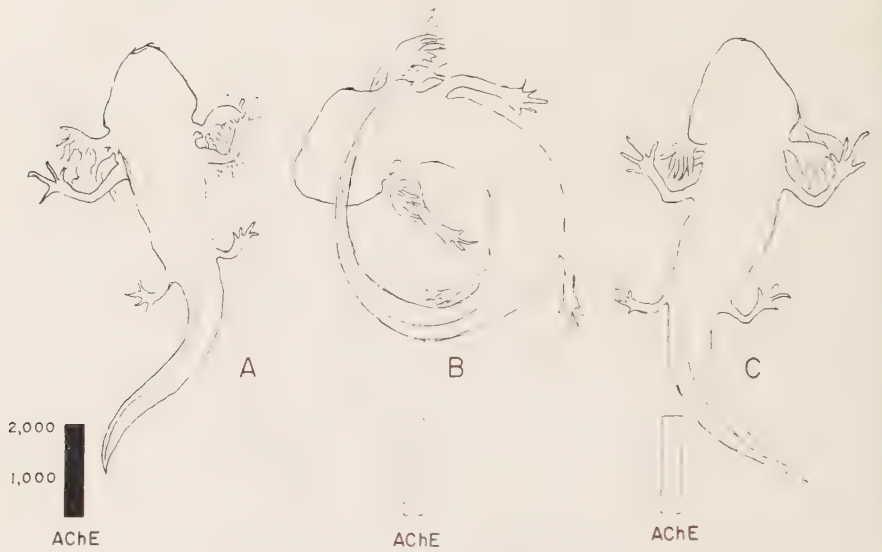

FIGURE 6. Effect of DFP on larval "overt behavior" and acetocholinesterase (AChE) activity. Top row: *A. punctatum* larvae, Stage 60 (Glücksohn): *A*, control larva; *B*, larva kept for five days in 10^{-7} M DFP solution; *C*, same larva (*B*) four days following transfer into spring water. All drawings schematized. Bottom row: AChE activity in $mm.^3$ of CO_2 per 100 mgm. of larval tissues, per 30 minutes. The AChE activities represent averages, each obtained with six larvae or more (Karczmar and Koppanyi, 1953).

this brief excursion into the relationship between cholinesterases and the mechanism of action of the so-called anticholinesterases, that the "overt reactions" to pertinent agents seem to depend on the "detailed" emergence of something else rather than the appearance and orderly increase of a particular enzyme.

To return to an attempt toward an explanation of the change of "overt reactions" to the depressants with the larval development, it was interesting to us to note that the larval sensitivity to "cephalic" depressants such as the barbiturates increased more rapidly than that to less specific depressants such as ethyl alcohol (FIGURE 1). Another helpful fact was that the sensitivity to spinal and medullary stimulants changed, within the larval development, at about the same speed (Karczmar and Koppanyi, 1947). It was tempting to say that these phenomena depended upon the fact that the receptor sites within the more phylogenetically primitive parts of the central nervous system, the medulla and the spine, develop at similar slower rates and are overtaken by cephalization. Parenthetically, ultimate low cephalization of urodeles accounts for their lower sensitivity to the "cephalic" agents compared to that of mammals (*op. cit.*). Also pertinent to the problem are the observations of Coghill (1929), that the differentiation of the cerebral parts of the brain catches up with that of postfacial regions late within the development "of overt behavior" after the appearance of swimming. Coghill (1929) and Herrick (1948) showed also that the larval cortex matures late [*cf.* also Coghill (posthumous) and Watkins, 1943]. All these data, as well as the results of Detwiler (1944, 1945, 1948), point to progressive and possibly discontinuous cephalization.

The next step was naturally that of direct experimentation. Larval head anlagen were removed at Harrison stages 21 and 22. As soon as the operated larvae reached the motile stage, they were exposed to chloretone, acetanilid, and MS-222 from the stage of the first motility on (FIGURE 8). A few available operated larvae were exposed to pentobarbital sodium at somewhat later stages (suitable controls were employed). Subsequent histology established that cerebral hemispheres and diencephalon were missing (Detwiler, 1945). As can be seen from FIGURE 8, all the operated larvae reacted to nembutal similarly, the induction time of anesthesia increased greatly. What is more striking is that, in the case of chloretone, the operated larvae fell into two distinct groups: some larvae were moderately less sensitive to chloretone than the unoperated controls, while others showed a very greatly delayed induction time (FIGURE 8). Two points have to be made. In the first place, there was no apparent histological difference between these two groups of operated larvae; in the second place, when the operation drastically affected the "overt reaction," the difference increased greatly with larval development (FIGURE 8). This finding is in line with the data of Detwiler (1945), indicating that similar operations begin to affect the swimming of the larvae only at a relatively later stage. Finally, there was little change in sensitivity to MS-222 and to acetanilid. Similar data with reference to MS-222 have been obtained by Anognostis and Rugh (1948).

These experimental data seem to support the conclusions reached indirectly

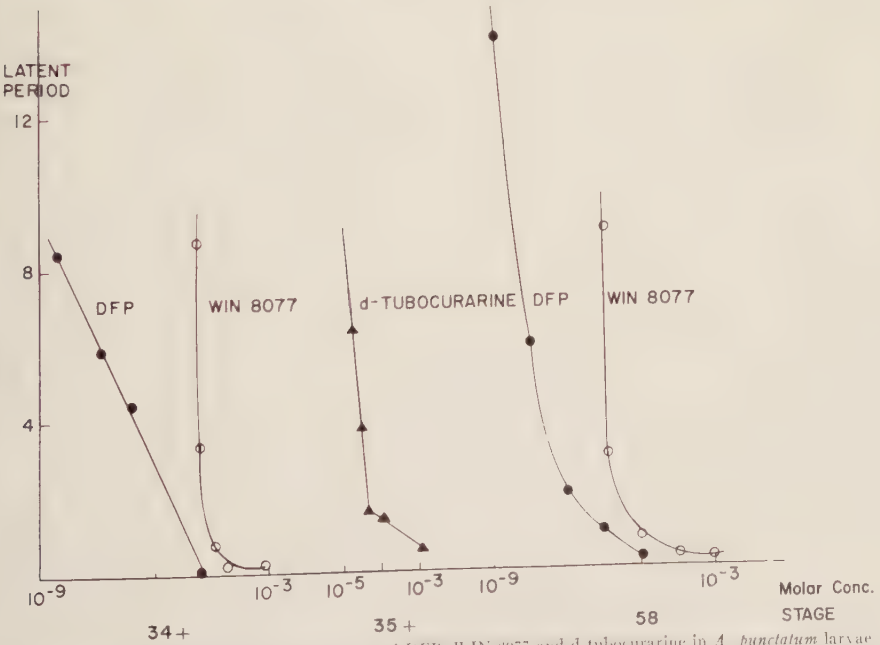

FIGURE 7. Latent period of "overt" effects of DFP, WIN 8077 and d tubocurarine in *A. punctatum* larvae. Ordinate: latent period in hours; abscissa: molar concentrations, logarithmic scale: 10^{-4} to 10^{-9} M for DFP and WIN 8077, Harrison Stage 34+; 10^{-3} to 10^{-5} M for d tubocurarine, Harrison Stage 35+; 10^{-3} to 10^{-9} M for DFP and WIN 8077, Harrison Stage 58.

that while the rapid change in the reactivity of the developing urodele larvae to certain "cephalic" depressants may be due to the more rapid development of the higher parts of the central nervous system, the slow or absent change of sensitivity to MS-222 and acetanilid is due to the slower change of the lower segments. It is noteworthy also that FIGURE 8 indicates that, although the discontinuous change in the sensitivity of developing larvae to the "cephalic" depressants may be absent after the operation, the slower continuous change is still present (experimental group 1, FIGURE 8). It may be hypothesized, therefore, that the operation brings the "cephalic" and the "lower" depressants to a common denominator. Thus, the action of the latter may depend entirely on some generalized receptors within the central nervous system. The "cephalic" depressants may have a similar component of action, but they have also another that can become effective with the differentiation of new specialized "cephalic" receptors.

How, then, may we explain the progressive change in larval sensitivity to "lower" depressants? As pointed out above, the heteroauxesis of the central nervous system with relation to other tissues is not an established fact. Some other possibilities, however, may be worth considering: the affinity of the drug

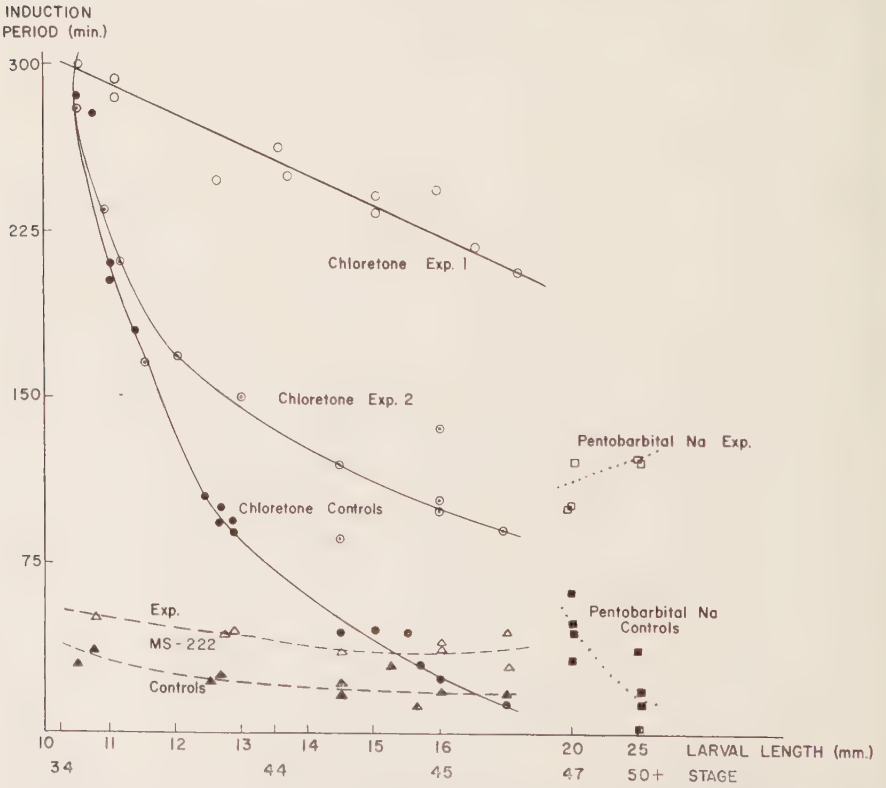

FIGURE 8. Comparison between the developmental change of the length of the anesthetic induction period of the control and of the decerebrated *A. punctatum* larvae. Legends as in FIGURE 2. See also text.

to the receptors may increase with development; or, with increased interneural coupling, less receptors may have to be depressed to interrupt the functioning of the central nervous system and induce anesthesia; or, finally, there is an increase of the overall efficiency (and, accordingly, of the sensitivity) of the enzyme systems supplying the energy for the "wakeful" central nervous system. There is some indication that the third possibility may, at least, play a part in the gradual increase of larval sensitivity to the "lower" depressants.

It is obvious that experiments presented here are still preliminary. The only safe conclusion, at present, is that "overt reactions" to certain drugs depend on differentiation of certain sites within the central nervous system. It is just possible that these sites do not correspond to histogenesis seen by standard methods. Certainly, in some cases, there must be differences in the material bases of "overt reactions" as demonstrated by the difference in the latter (FIGURES 2 and 8), which cannot be demonstrated at present by any other methods. Finally, it has been also pointed out that, in the case of the larval "overt reactions" to the anticholinesterase agents, the changes in the enzyme activity could not entirely explain the results.

*Effects of Certain Carcinogens on Regeneration**

In the case of larval limb regeneration, what could be otherwise regarded as the repetition of limb ontogeny is additionally complicated by the already present limb organization. The embryonic "recapitulation" (Schotté, 1940) cannot begin until the cellular proliferation with the intervening controversial process of dedifferentiation of the larval stump tissues prepares the blastema. Dedifferentiation was, and may still be, in the position of the nineteeth century ether, *i.e.*, its presence is necessary to explain certain phenomena, but no direct proof of its existence is forthcoming. Indeed, most agents used by the Amherst-Princeton School of Investigators (Schotté, 1922; Butler, 1933) and their spiritual heirs seemed to lead invariably to regression (Lehman, 1947; Thornton, 1943, 1950; Karczmar, 1948), which was explained as continuous dedifferentiation with underlying "disposal" (Schotté and Karczmar, 1944, 1945). Obviously, however, regression lacks more than one element of normal regeneration. There is neither proliferation nor differentiation and, thus, regression is not a "pure phase" obtained by inhibiting just one of the many regenerative components normally contributing to the regenerative cellular equilibrium (Butler and Schotté, 1941, 1949). Schotté and Butler recognized this point by stating that nerves are required for (1) the accumulation phase of blastema formation and (2) the onset of induction processes in the blastema (1944). Moreover, accumulation blastema has the potency of checking regression (Schotté, Butler, and Hood, 1941). An additional function of nerves was postulated by Rose (1948). However, on the basis of this very division of phenomena leading to regeneration, one would expect theoretically that there could be induction without cellular proliferation and accumulation, and that induction could be combined with regression and dedifferentiation.

This theoretical expectation seemed, at first, justified when Doctor G. Berg

* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institute of Health, United States Public Health Service, Bethesda, Md.

FIGURE 9. "Dwarf" resulting from belated transfer of DBAS-treated larva to spring water. *A. punctatum*, Stage 60+, was exposed for four days after amputation to 32 mg. per cent of DBAS and then transferred to spring water. Regression persisted for nine days thereafter (rates −1.1 and −0.5 μ/hr. for the left and right limb). Elongation was resumed 13 days after amputation (rates from 13th to 27th day, 2.6 and 2.1 μ/hr.). Larva was fixed on the 28th day of the experiment. (*a*) The regenerates on the 24th day of the experiment; (*b*) histology of the right regenerate (28th day). A photographic mosaic of six serial sections. The humerus, partially resorbed during the initial regression, shows now a primordium-like stacking up of the cartilage cells (Bouin fixative, longitudinal 6 μ sections, Heidenhain-Eosin stain, ×50). Karczmar and Berg, 1952. In FIGURES 9 to 12 and in PLATE 1, DBAS refers to disodium 1,2,5,6-dibenzanthracene trans-endo-α,β succinate, and MC to 20-methylcholanthrene.

and myself could show that the carcinogens, methylcholanthrene, and dibenzanthracene, could produce in Stage 60 (Glücksohn, 1931) *A. punctatum* larvae "dwarfed" regenerates (FIGURE 9) or even regression (in the case of relatively strong dibenzanthracene solutions) with little or no effect on differentiation (Karczmar and Berg, 1952, p. 513, plate 1). Moreover, at no concentration compatible with life, could differentiation be affected. The regenerates so obtained (FIGURE 10) were called by us "regressed dwarfs." Interestingly

enough, the changes that led to their formation were not, apparently, irreversible (FIGURE 11). Further data seemed less clear cut:

(1) Dibenzanthracene did affect differentiation provided it was applied not from the moment of amputation on, but beginning only at later regenerative stages (FIGURE 12). This was a paradoxical situation, since it appeared that the carcinogen applied throughout regeneration was less active, in a sense, than when applied for shorter time periods.

(2) Dibenzanthracene could affect also the regenerative differentiation of younger larvae (Glücksohn's Stage 52) and of the larvae of another species (*Pseudotriton ruber*). In many such cases, straight regression developed (PLATE 1).

(3) Further difficulty arose when a study of the "regressed dwarfs" revealed that their formation was accompanied by some mitosis and by histogenesis resembling normal regeneration and blastema formation rather than regression. This phenomenon was somewhat unexpected from the standpoint of the as-

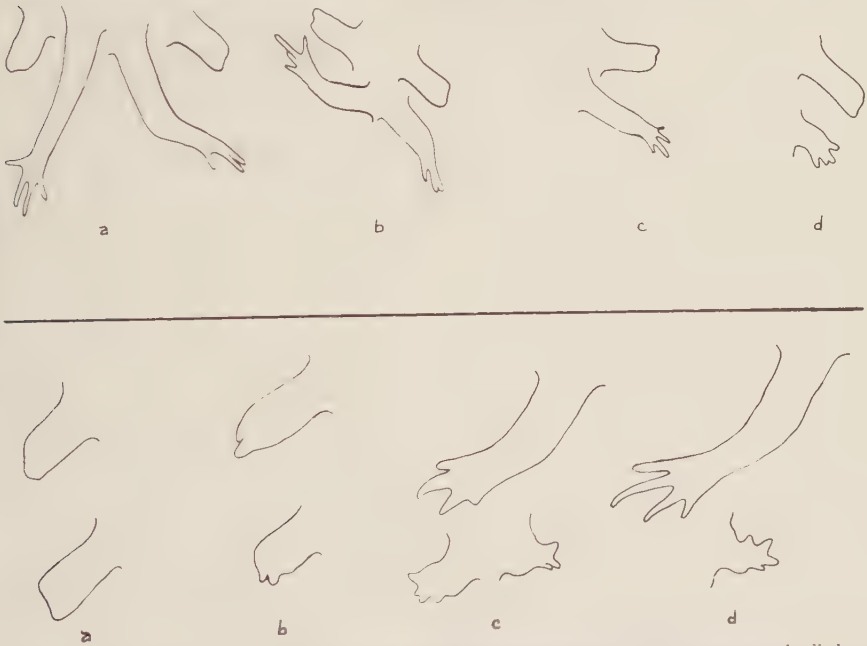

FIGURE 10. Top row: effects of MC injection and different concentrations of DBAS on regenerating limbs of *A. punctatum* larvae, Stage 60+. Camera lucida drawings, magnification × 17.5. (Drawings show ventral aspect in *a* and *b*, right limbs are shown at the left; left limbs are on the right; the aspect of the limb immediately after amputation shown first.) *a*: Control case reared in spring water one month after amputation. Elongation rates: 4.7 μ/hr. for the left and 4.1 μ/hr. for the right limb. *b*: MC injected case three months after amputation. Elongation rates after 700 hours: 1.2 μ/hr. for the left and 1.6 μ/hr. for the right limb. *c*: DBAS treated case, four months after amputation and three months after transfer from 16 mg. per cent DBAS to spring water. Left limb only shown. Elongation rate after 700 hours: 0.9 μ/hr. (*d*) DBAS case on the 41st day of exposure to 40 mg. per cent DBAS. Left limb only shown. Regression rate 0.4 μ/hr. Bottom row: Synchronization of morphogenetic stages in regeneration of controls and treated (40 mg. per cent DBAS) Stage 60+ *Amblystoma punctatum* larvae. Camera lucida drawings, magnification ×20. Control case—top; experimental case—bottom. (*a*) Left stumps two days after amputation. *b*: Two finger buds stage 14 days after amputation. (*c*) Both right limbs (control and experimental) exhibit, 27 days after amputation, three digits and a finger bud; left experimental limb—three digits only. *d*: Five days later, the left experimental limb shows four digits. From Karczmar and Berg, 1952.

FIGURE 11. Regenerate of a reamputated "dwarf," exposed for one month to 40 mg. per cent of DBAS. The larva was then transferred to pond water for two months and subsequently reamputated through the metacarpal region. Forty two days after reamputation, the regenerate had three normal size fingers on a "dwarf" arm. The section shows portions of elongated digits and a dwarfed humerus and ulna. Bouin fixative, longitudinal 6 μ sections, Heidenhain-Eosin stain, 32 mm. objective). From Karczmar and Berg, 1952.

sumption outlined above that the induction may proceed in the absence of cellular accumulation.

Is there any reconciling of these data? Let us say, first, that the carcinogens are relatively mild inhibitors of regeneration. In their presence, "some formation of blastema can proceed, due to the processes of dedifferentiation, cell migration, and even limited mitosis" (Karczmar and Berg, 1952, p. 505). Moreover, we have data demonstrating clearly that agents used hitherto to inhibit regeneration must do more than just suppress blastema formation. One would expect, for instance, that a cytotoxic agent such as nitrogen mustard, which completely suppresses cellular proliferation and blastema formation (Karczmar, 1948), would cause at least as rapid a regression as denervation. Actually, nitrogen mustard regression is quite slow, while denervation and exposure to nitrogen mustard cause the fastest regression recorded as yet (Karczmar and Berg, 1950a). It seems clear that regression is a composite process, and that any single agent may act on only one or a few, but not on all of its components. However, all hitherto tried antimitotics as well as denervation have one thing in common: they suppress the morphogenesis. Do we have to ascribe this effect to their direct inhibition of differentiation? It is more likely that the capacity for differentiation is inherent in the "cellular equilibrium" (Butler and Puckett, 1940) represented by the late blastema and

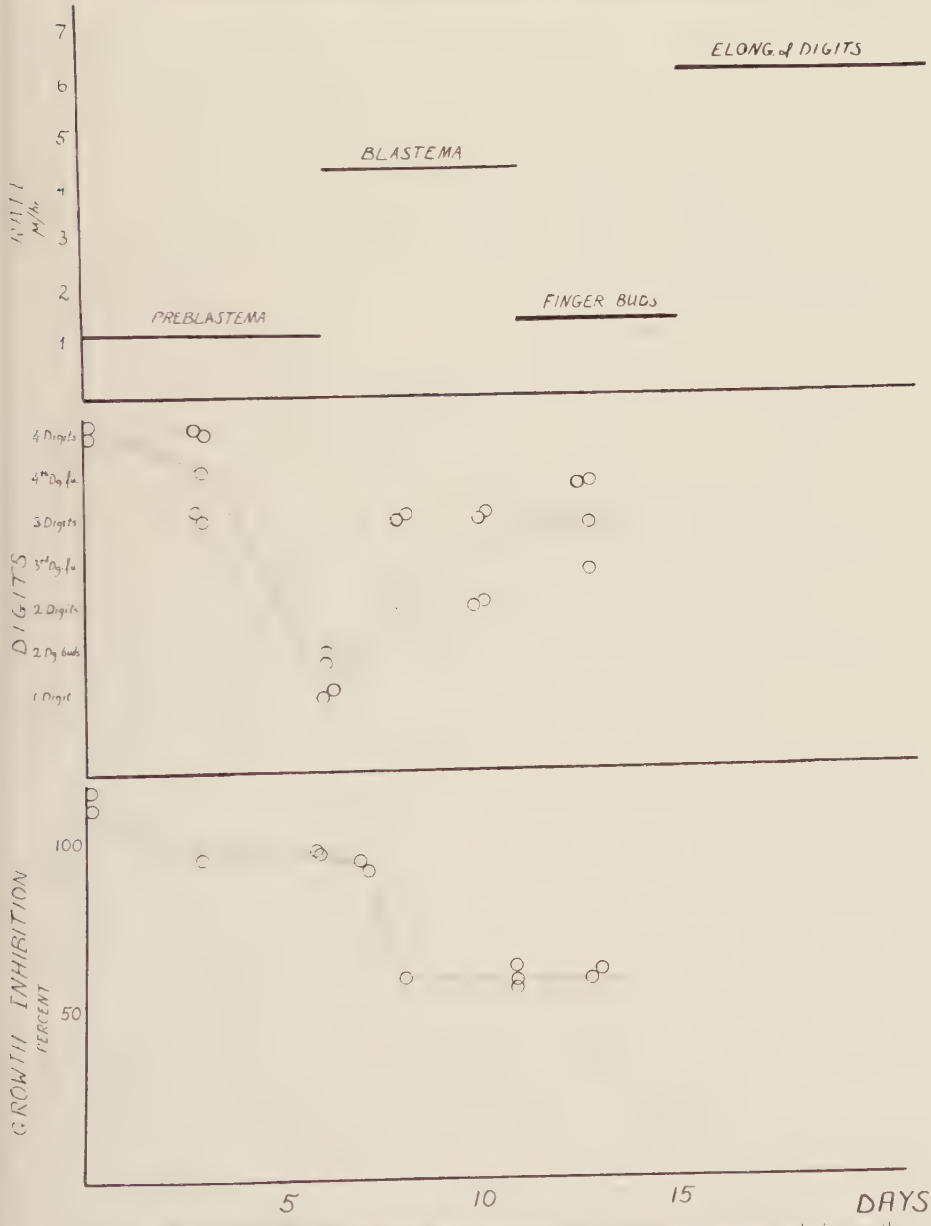

FIGURE 12. Effect of exposure at successive regenerative stages to DBAS, .2 mgm. per cent solution, on the rate of elongation and on differentiation of amputated *A. punctatum* limbs. Ordinate: top—average elongation rates of control regenerates during the three regenerative phases indicated; middle—extent of digit differentiation obtained after DBAS (40 mg. per cent) treatment initiated at regenerative periods indicated by the empty circles; bottom—inhibition of elongation in per cent. Abscissa: days after amputation.

certainly dependent on many components. When this equilibrium is upset by interfering with one or more of its components (thus, for instance, denervation may affect more than one of them, see above) differentiation will be inhibited, even if the agent in question has no antidifferentiative action. On the other hand, differentiation may be specifically inhibited. In other words, not only should there be, theoretically, differentiation without growth and elongation, but also elongation and growth without differentiation. Actually, some arrested blastema were seen by Schotté and Butler (1944), Singer and Craven (1948), and by myself (1946), differentiation being inhibited to a varying degree. It may appear further that, although dedifferentiation during regression can certainly lead to an accumulation of cells comparable in volume and cell number to that of a blastema, some inductive property is missing. This phenomenon may occur because of the antiinductive effect of denervation (Schotté and Butler, 1944) or because a factor, present in a proliferating blastema, is missing in regression. It is thus possible that not only carcinogens leave some of the components of the equilibrium undisturbed, but also allow some cellular proliferation to occur. Thus, the missing factor of the regenerative equilibrium is now present.

As a matter of fact, it seems that no new equilibrium is possible with antimitotics or after denervation, and thus regression ensues. On the other hand, a new equilibrium is possible in regenerates of the Stage 60 *A. punctatum* larvae in the presence of dibenzanthracene, although it may not be achieved at an earlier stage or in another species. Indeed, "the presence of this new equilibrium may explain why even the smallest 'regressed dwarfs' do not fall below a certain size . . . . . they seem to be a fairly constant fraction of the normal regenerate . . . . . , thus, the new equilibrium* results in a specific size of the 'dwarfs' just as a normal blastema leads to a regenerate of normal size" (Karczmar and Berg, 1952, p. 505). I think that here are also the bases of an explanation of the antidifferentiative effect of carcinogens applied at later regenerative phases of the Stage 60 *A. punctatum* (see above). Since no experiments are available in support of such an explanation, the argument will not be pursued at present.

Finally, since some mitoses and cellular accumulation accompanies the formation of "regressed dwarfs," we obviously cannot claim that the latter constitute, at last, an example of differentiation arising from entirely dedifferentiated modulated (Weiss, 1939) tissues. Let me state, however, that regression as we know it from regressing denervated or irradiated stumps may give us a picture of dedifferentiation which is somewhat distorted by the cytolytic processes initiated by the agents used. It is quite likely that a more biological picture of dedifferentiation is that obtained by the use of obviously milder acting agents such as carcinogens. As a matter of fact, their action is so mild that the processes of carcinogen-treated regeneration (at least in the case of the Stage 60 *A. punctatum* larvae) are not easily distinguished, on the histologic level, from those of normal regeneration; yet their "overt" result, the "re-

* I do not think that either Doctor Butler (who first spoke of the cellular equilibrium in the blastema) or ourselves consider it to be a resultant of only local influences. Indeed, recent results of Schotté (personal communication) indicate that this equilibrium is as much under systemic as under local control.

gressed dwarf" instead of the full size regenerate, is very different. Apparently, the "overt" reaction of regeneration to such chemical agents as carcinogens is a more sensitive indication of the underlying components of the process than other available techniques (this statement was already made in this paper in another connection). It may be that their effect, confined seemingly to the suppression of elongation, is concerned with physical bases of differentiation such as cellular migration and disposal processes already assigned on other grounds an important role in regression and regeneration (Schotté and Karczmar, 1944, 1945; Karczmar, 1946). Thus, the real mechanism of their effect may escape our methods, at present, as it should appear also from the last section of this paper.

Some Metabolic Processes of Regeneration

If dedifferentiation escaped, at least partially, the proof of its existence by analyzing the phenomenon of "regressed dwarfs," it was hoped that there may be a way of characterizing the cells in question so as to identify them with a known cell strain. By definition, dedifferentiation is a cellular change. It is thus differentiation in a contraontogenic direction.

Ontogenic differentiation was associated with an increase of alkaline phosphatase activity (Moog, 1944; Brachet, 1946; Krugelis, 1950). We could add to these data the histochemical measurements of alkaline activity in ontogeny of the larval salamander limb (PLATE 2; Karczmar and Berg, 1951). Also, in this case, the cells of early primordium are characterized by a strong phosphatase stain appearing first in the nuclei and subsequently in the cell. The height of the enzyme activity appears early in histogenesis but, subsequently, the differentiating tissues show first loci and then general areas of the decrement of the alkaline phosphatase staining. The question that we can ask now is: When would the alkaline phosphatase stain, apparently characteristic for cells undergoing change of fate, arise in the course of regeneration? During regenerative differentiation or perhaps during dedifferentiation?

A histochemical study of alkaline phosphatase in normally regenerating *A. punctatum* limbs (*op. cit.*; PLATE 2; FIGURE 13) shows, first of all, a latent period of three to four days following the amputation. Subsequently, the cells of the early blastema begin to stain strongly. The blastema is a site of strong enzymatic activity (PLATE 2; FIGURE 13) but, as in the ontogenic limb, the phosphatase activity is on the wane at the moment of the beginning of histogenesis. FIGURE 13 illustrates two other interesting points. Specialized cells (calcifying perichondrium and cartilage, and also certain areas in the hypodermis) stain strongly even in the generally phosphatase-poor larval limb. It is not difficult to differentiate between this functional enzyme and the enzyme in ontogeny or regeneration. Second, phosphatase-rich mesenchyme seems to be a transition point both in ontogeny and in regeneration. Parenthetically, two strains of fibroblasts were found prior to amputation, one at a low and another at a high phosphatase activity level. While the former had to increase its phosphatase activity during early regeneration (FIGURE 13), the latter could, so to speak, calmly await the events.

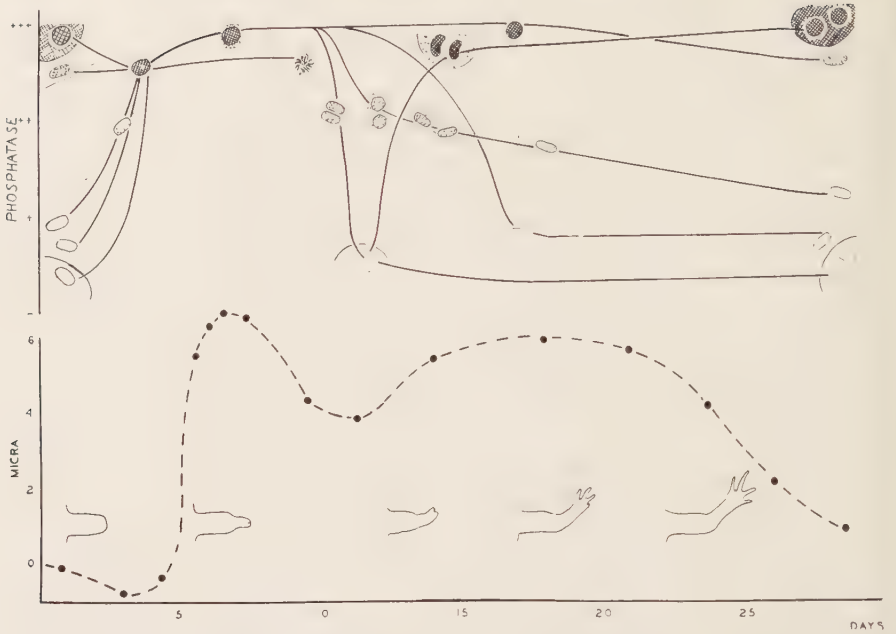

FIGURE 13. Relation between rates of limb regeneration and alkaline phosphatase activity in cell strains. The histological and histochemical changes are presented schematically for five tissues, from top to bottom: hypertrophic cartilage, perichondrial (or hypodermal) fibroblast, muscle, connective tissue fibroblast and hyaline cartilage. Note that fibroblasts can begin their postamputation changes either at a low or high level of alkaline phosphatase. The stages of limb regeneration are represented by camera lucida drawings at the bottom of the figure. Abscissa: days after amputation; lower ordinate: rates of change in limb length in micra per hour; upper ordinate: alkaline phosphatase activity on a four unit scale. Density of stippling indicates depth of stain. From Karczmar and Berg, 1951.

With reference to the problem that interests us at present, a high phosphatase level is clearly visible at about the fourth day after amputation (20° C.), *i.e.*, at the stages assigned by many investigators to dedifferentiation. Maturation and differentiation of the blastema occurs only a week or more later. We can say, therefore, that the cells of the preblastema and early blastema are actively engaged in a process probably associated with a high rate of phosphate turnover. Since cellular phosphatase does not appear to be associated with mitotic activity (*op. cit.*) and, since early embryonic, proliferating cells are phosphatase-poor until differentiation sets in (Krugelis, *op. cit.*), the argument can be put up that alkaline phosphatase activity characterizes the change in the cellular fate, and that such a change occurs long prior to regenerative differentiation.

A further extension of this argument can be produced by showing that nonregenerating regressing limbs are also phosphatase-rich, as rich as the limbs that will subsequently differentiate (FIGURE 14; Karczmar and Berg, 1950a and 1950b). Moreover, as can be additionally seen, some preliminary measurements of the oxygen uptake of regenerating and nonregenerating larvae show, in both cases, a marked increase at the relatively early preblastema stages (Karczmar, unpublished). Thus, *dedifferentiating* limbs resemble ontogenic *differentiating* limbs by their high metabolic activity. The phosphatase-rich

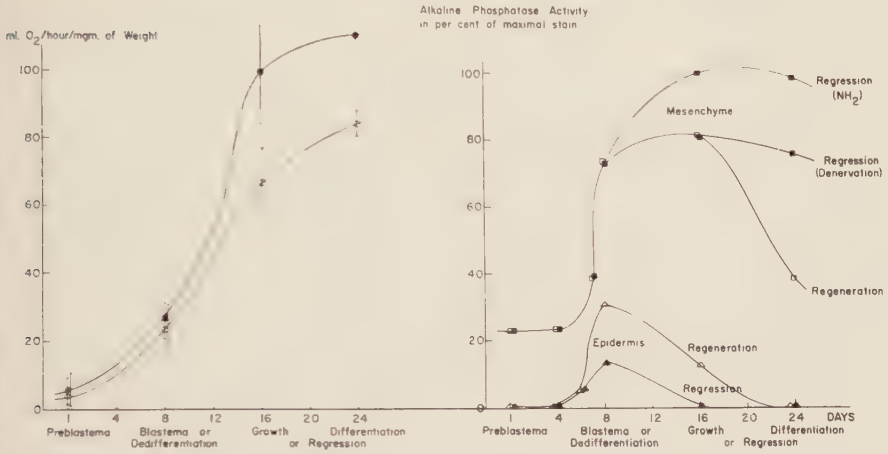

FIGURE 14. Oxygen uptake and alkaline phosphatase activity in regenerating and regressing limbs of *A. punctatum* larvae. Stage 60+ (Glücksohn). Ordinate: oxygen uptake in μl O_2/hour/mg. of limb weight and alkaline phosphatase activity in per cent of maximal stain as developed in certain reference structures (*cf.* Karczmar and Berg, 1951, p. 141). Abscissa: days after amputation and stages of regeneration or regression. Other legends in the figure are self-explanatory.

dedifferentiating mesenchyme appears to be homologous to the ontogenic mesenchyme ready to differentiate.

This similarity seems to me to constitute a strong argument for considering dedifferentiation as representing a distinct change in cellular fate, characterized by a high energy level. Parenthetically, it should be remembered that, 13 years ago, the extreme dedifferentiation shown by Butler and Schotté (1941) to occur in denervated limbs was considered by some as necrotic in nature.

The problem arises, however, as to whether the change in enzyme activity is the earliest characterization of the process in question. Indubitably, this inference is not valid, as far as the measureable events are concerned. Alkaline phosphatase, as well as the increase in respiration, both can be first measured days after the amputation has released the regenerative stimulus. Of course, it can be argued that our histochemical and biochemical methods are not sensitive enough to show early increments in the pertinent metabolism. Additionally, however, the measurements of metabolism are not sufficiently sensitive in another sense also, as is obvious from FIGURE 14. On the basis of the two metabolic changes presented in this figure, it would be difficult to differentiate, up to eight days after amputation, between regeneration and regression. Yet, even without stressing the obvious, namely, that the difference between the two is great, let me repeat that the use of appropriate chemical agents allows us, as shown above, to separate components of these two processes and wind up with a wide range of responses from slow to rapid regression without differentiation, and from rapidly elongating regenerate to a regressing regenerate (Karczmar, 1946). In this case, at least, the responses to the chemical agents proved more specific than the measurements of, admittedly very few, metabolic activities.

I have tried to point out some of the difficulties in relating the problems of "overt behavior" to enzymes. It is obvious that the same difficulty exists

also in regeneration. I should like also to refer in this context to some experiments of Grobstein (1947, 1948). Apparently, the castrated males and females of *Platypoecilus maculatus* regenerate a gonopodium when exposed to methyl testosterone. Even with the optimal concentrations, however, morphogenesis "differs significantly in temporal pattern and details of final morphology" (Grobstein, 1948, p. 232) from that of the normal male. In similar fashion, Schotté argues that the pituitary controls only the "epiphenomenon" of regeneration: it is "instrumental solely in facilitating the 'regenerated epidermis-cut tissues' relationship indispensable for the onset of regeneration" (Schotté and Hall, 1952, p. 555). Although future experiments may show that a hormone was lacking in Grobstein's medium and that other hormones are more essential to regeneration than the pituitary, it may be also that, in all these related problems, the hormonal and metabolic characterization of the events is secondary only to the basic cellular and molecular control of the pertinent processes.

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PLATES

PLATE 1 (from Karczmar and Berg, 1952). Nos. 1 to 5. Histology of partially and totally inhibited limbs of *A. punctatum* larvae under DBAS treatment. Larval Stage 60+ except for No. 5. (Bouin fixative, longitudinal 6 μ sections, Heidenhain-Eosin stain, $\times$ 50.)

No. 1. Nine-day regenerate regressing rapidly (2.2 μ/hr.) in 40 mg. per cent DBAS. Distal end of the humerus collapses; there is some accumulation of mesenchyme.

No. 2. Strongly inhibited regenerate after 11 days of exposure to 24 mg. per cent DBAS. Early finger bud stage, elongation rate: 0.3 μ/hr.

No. 3. Rapidly regressing dwarf after 21 days of exposure to 40 mg. per cent DBAS (regression rate: 1.3 μ hr.). Two of the three finger buds present are shown in this section. Note the remnants of humerus in regressing upper arm.

No. 4. Partial inhibition of growth with 16 mg. per cent DBAS. Right regenerate 25 days after amputation (elongation rate: 0.6 μ/hr.). Dwarfed elbow, forearm and wrist in a three-finger stage.

No. 5. Partial inhibition of growth by 8 mg. per cent DBAS. Left regenerate (stage 52, *A. punctatum* larva) 24 days after amputation (elongation rate: 0.9 μ hr.). Dwarfed elbow, forearm, and wrist in a permanent three-finger stage.

Nos. 6 to 8. Histology of total inhibition by DBAS of limb regeneration in *P. ruber* larvae. (Bouin fixative, longitudinal 6 μ sections, Heidenhain-Eosin stain. $\times$ 100.)

No. 6. Early inhibition with 40 mg. per cent DBAS. Slow regression (0.1 μ hr.) on the 15th day.

No. 7. Total inhibition with 40 mg. per cent DBAS. Slow regression (0.2 μ hr.) on the 28th day. Mesenchyme at tip with mitoses.

No. 8. Total inhibition with 24 mg. per cent DBAS. Slow regression (10.2 μ/hr.) on the 38th day. Note accumulation of mesenchymal cells.

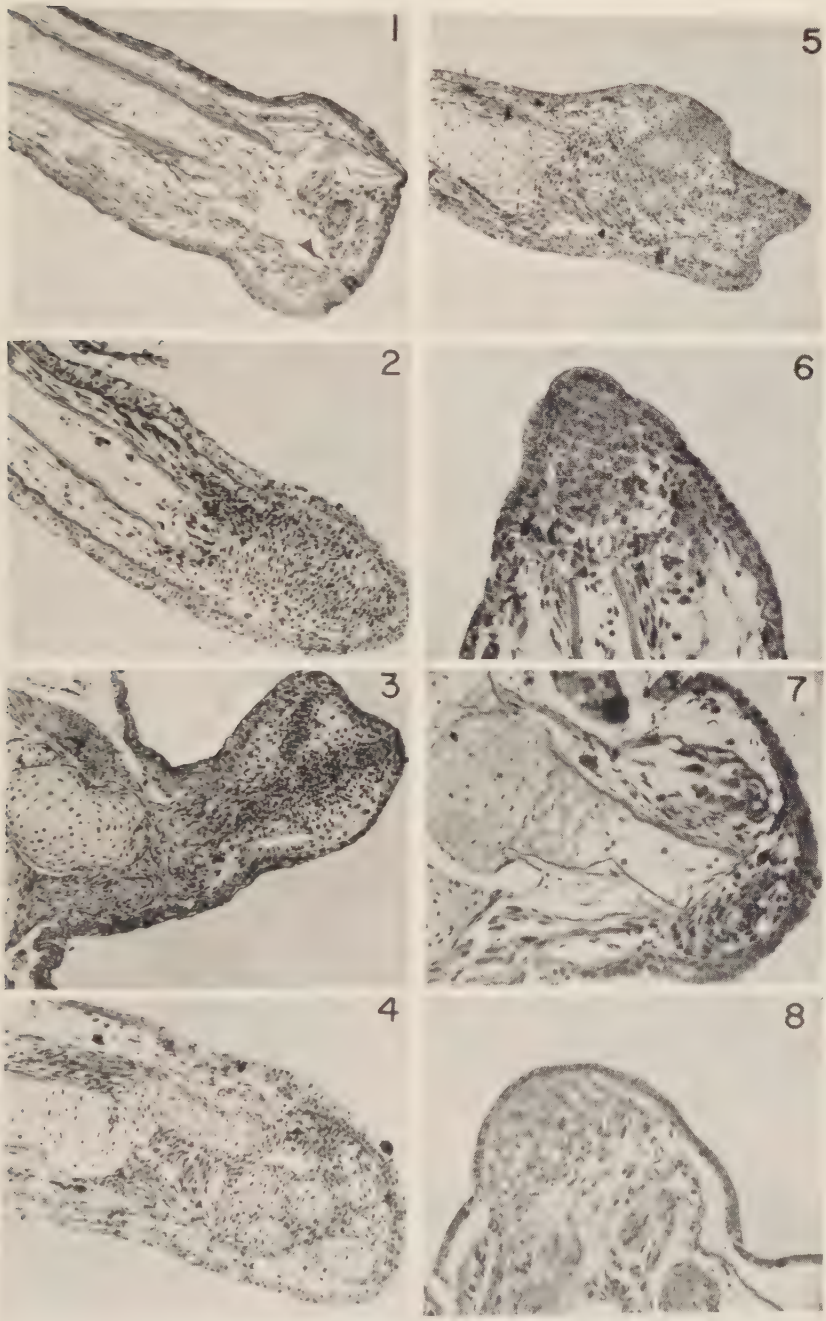
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PLATE 2 (from Karczmar and Berg, 1951). Nos. 1 to 5. *Amblystoma punctatum* larvae (staged after Glücksohn, 1931): 6μ sections stained for alkaline phosphatase.

No. 1. Phosphatase gradient in the early limb bud. Ventral plane section through a hind limb bud and cloaca of a Stage 50 larva. The apex of the limb primordium, and some gut contents, stain +++. *G*, lumen of cloaca. ×20.

No. 2. Phosphatase activity in the ontogeny of organ fields. Longitudinal section through hind limb bud and base of tail, Stage 50. *H*, upper leg center of enzyme activity (+++); *A*, presumptive foot field (++ +); *Mh*, myoblasts of the upper leg (++). ×80.

No. 3. Phosphatase in primary ontogenic differentiation. Longitudinal section through the region of the fibula, Stage 52. Chondroblasts (*C*) stain ++; early myofibrils (*M*) stain + to ++. ×360.

No. 4. Phosphatase distribution in histogenesis. Longitudinal section through the hind limb and flank, Stage 54. Progressive loss of enzyme from + + to + is shown in three stages of muscle differentiation (*M*). Differentiating and body hypodermis (*H*) stains +++. *D*, matrix of derma (−). ×20.

No. 5. Detail of FIGURE 4: fibula and knee. Progressive proximodistal loss of enzyme from + + + to + is seen in the chondrocytes of the bone. ×360.

Nos. 6 to 10. *Amblystoma punctatum* larvae; 6μ sections of regenerating forelimbs, stained for alkaline phosphatase.

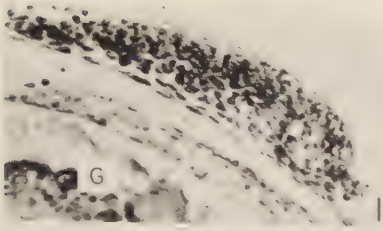
No. 6. Normal enzyme distribution. Healing on the first day after amputation. *E*, epidermal cap (−); *M*, muscle (+); *C*, diaphysis and marrow of humerus (++); *H*, hypodermis. ×150.

No. 7. Dedifferentiation and first increase in enzyme activity. third day after amputation. Hypodermis *H*, perichondrium (*P*) and weakly staining muscle (*M*) dedifferentiating distally into + + mesenchyme.

No. 8. Accumulation of enzyme rich mesenchyme: 5th day after amputation. *Bm*, mesenchyme, staining ++ to + + +; *C*, proximal epiphysis of humerus with + ++ matrix; *Ch*, hyaline cartilage of shoulder joint with enzyme free matrix; *M*, muscle (+); *H*, hypodermis (+++). ×80.

No. 9. Conical blastema, staining + + +, 10 days after amputation. This section through the margin of the perichondrial sheath of the humerus (*P*, + +) demonstrates the enzyme distribution in muscle. *H*, hypodermis (+ + +); *D*, enzyme free distal edge of collagenous derma marking the level of amputation; *Ch*, hyaline cartilage, with (−) matrix. Note the patches of enzyme activity in the epdermis. ×20.

No. 10. Detail of a differentiating blastema, 12 days after amputation: *C*, rudiment of humerus, illustrating from right to left, the primary differentiation of chondrocytes accompanied by a drop of enzyme activity from + + + to +, and the secondary increase of intracellular and extracellular enzyme activity in the hypertropic chondrocytes; *M*, differentiating muscle, staining + +; *P*, differentiating perochondrium staining + + +. *V*, blood vessels with + ++ walls.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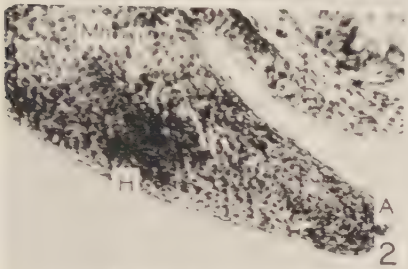
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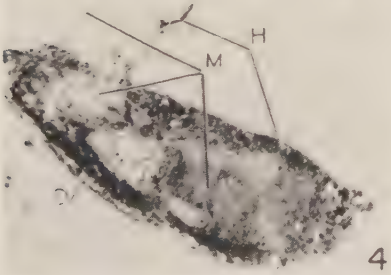
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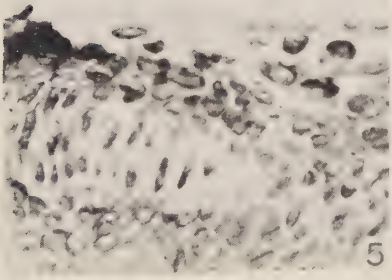
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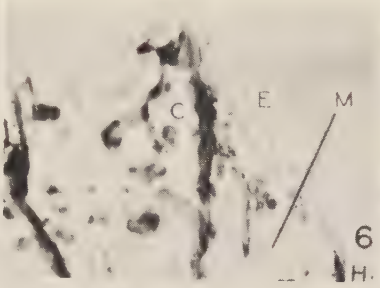
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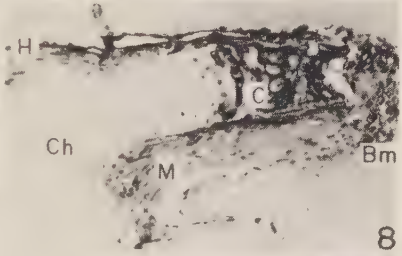
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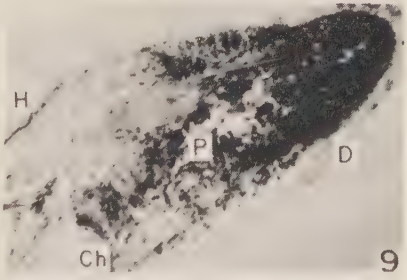
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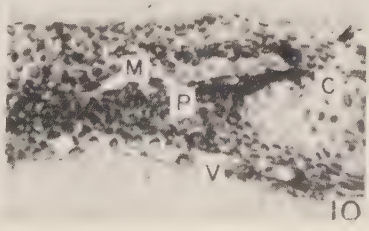
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SPECIFIC INHIBITION DURING DIFFERENTIATION

By S. Meryl Rose

University of Illinois, Champaign, Ill., and Marine Biological Laboratory, Woods Hole, Mass.

The hypothesis (Rose, 1952) that differentiation proceeds as more rapidly developing regions suppress similar development in more slowly developing regions is being tested. A cardinal feature of this view is that the slower regions, prevented from forming those structures already differentiating adjacent to them, are still able to form other structures and thereby complete the pattern of a whole.

The work reported here consists of two parts. In the first, by combining parts of older and younger regenerating *Tubularia*, we are able to test whether an older part can suppress the differentiation of a younger like part and thereby force it farther down on the ladder of differentiation. In the second part, the evidence for specific inhibition of differentiation of embryonic organs in frog embryos by products of the like adult organs is presented.

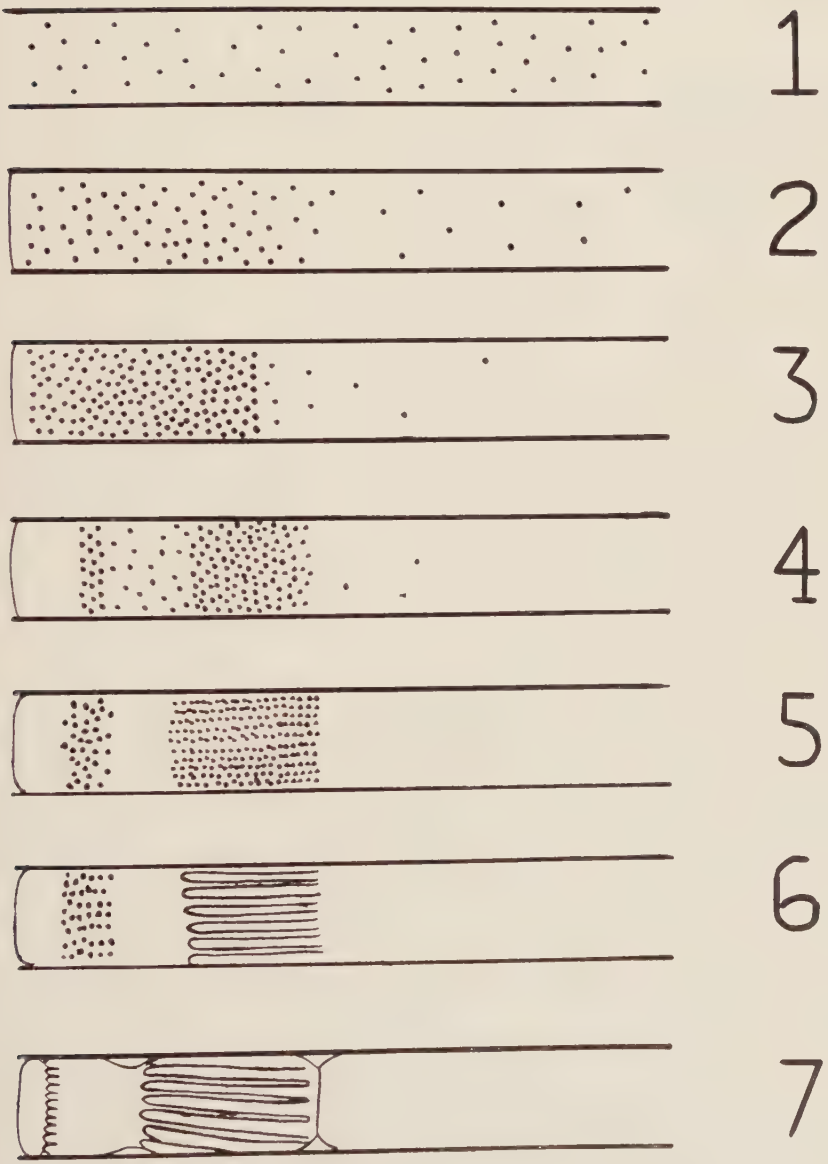
Tubularia

The first thing which had to be determined with the regenerating *Tubularia* was the direction in which differentiation proceeds. From the extensive work reported by Child (1941) one could be quite sure that differentiation would begin in the distal region and proceed proximally. In the case of *Tubularia*, however, there is the distracting fact that one can see proximal tentacle formation before distal tentacles have begun to appear (CHART 1).

Using the test of isolation, one learns that distal parts are capable of self-differentiation by the time tissue has concentrated at the regenerating end of the stem (stage 2). Such pieces form very little more of a hydranth than if they had been left in place as part of the whole. There is very limited regulation in the proximal direction. In some cases, gonophores and a few proximal tentacles may develop in the distal isolates demonstrating a slight tendency toward proximal completion. The data are summarized in TABLE 1.

The proximal part of a regenerating primordium behaves differently upon isolation. It shows an instability which prevents one from obtaining an answer to the question, "Is it determined?" Either it loses its former organization completely and reorganizes or, without extensive loss of organization, it begins to complete itself distally. In over half of the cases there was complete loss of form already attained in this region. Proximal tentacle striations fade. Many cells lose their positions in the peripheral coenosarc, and almost complete disaggregation ensues, the gastrovascular cavity becoming opaque with cells. After disaggregation, the process of regeneration can begin again, but the result is never just a proximal part of a hydranth. Once the tissue has lost its former organization, the cells have no "memory" of their former proximal nature. If the disaggregates recover and regenerate, they always form distal structures. The most frequent type of regenerate is the bipolar distal type (CHART 2). In this type, distal structures arise at both ends, and there is insufficient material

CHART 1
STAGES IN RECONSTITUTION



(1) Freshly cut stem
(2) Pigment concentrating in future hydranth region
(3) Pigmented primordium
(4) Two pigment bands.
(5) Proximal band striated.
(6) Distal band striated, proximal tentacle ridges
(7) Distal and proximal tentacles, hydranth delimited at base

Table 1

Structures Formed in Isolates of Regenerating Primordia (Stages 2 to 7)

	Distal isolates	Proximal isolates	Proximal isolates and stem
Hypostome and distal tentacles	27	0	0
Hypostome, distal tentacles and gonophores	6	0	0
Hypostome, distal tentacles, gonophores, and a few proximal tentacles	5	0	0
Proximal region only	0	3*	0
Complete hydranth	0	1	19
Regression followed by production of distal structures only	2	8	0
Regresstion followed by production of complete hydranth	0	7	11
Regression without subsequent reorganization	2	2	4

* Only proximal isolates from stage 7 or later ever maintain their form even for a few days in the absence of distal structures.

in the center to complete a hydranth proximally. More or less of the proximal part of the hydranth is lacking. Many have no gonophores or proximal tentacles. Others have gonophores but no proximal tentacles. The smallest contain distal structures only. These small regions and small regenerates, in general, as observed by many investigators, always form distal parts. Only when there is sufficient material do the more proximal parts form. This development is in itself evidence that distal parts differentiate first, even though, in larger primordia, we may see proximal tentacles before the distal ones.

Not all proximal isolates of differentiating primordia suffer complete loss of organization. Some, after removal of distal portions, produce a new hypostome and distal tentacles with very little delay. Often a visibly differentiated proximal isolate will change only in its more distal portion and produce normal distal structures. But whether there is great or little reorganization, the result is always formation of a new distal region.

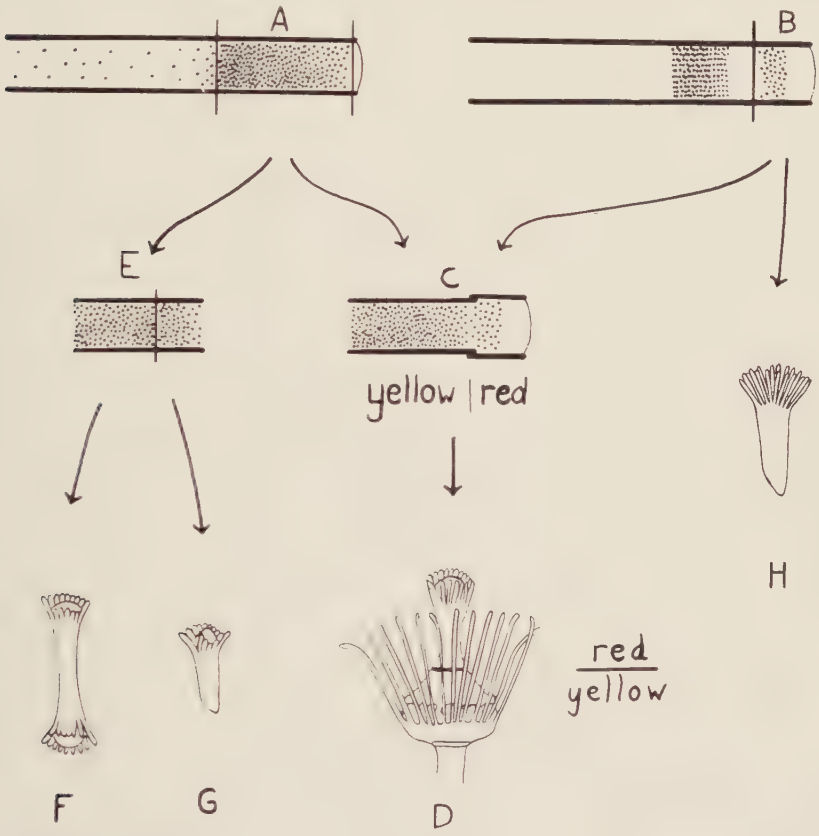
The above description is a confirmation of observations first made by Driesch (1897). In recent years, Davidson and Berrill (1948) and Rafferty (in press) have also demonstrated the unidirectional nature of reconstitution in fragments of primordia of *Tubularia*. In Rafferty's words, "It may therefore be a rule that fragments from polyps and polyp primordia can reconstitute structures that are normally distal to the original location of the fragments but not more proximal structures."

It would seem, then, that if organization is attained and maintained by suppression of the distal type of differentiation in more proximal regions, it must proceed in the distoproximal direction.

We are ready now for the crucial test of the hypothesis. If we were to combine an older and a younger distal region with the proper orientation during the differentiation phase, the older one should prevent the younger one from achieving the distal type of structure and leave open to it only the possibility of forming more proximal structures. In practice, an older distal region of a

CHART 2

DIAGRAM OF OPERATIONS AND TYPICAL RESULT WHEN AN OLDER DISTAL REGION IS GRAFTED TO THE DISTAL END OF A WHOLE YOUNGER PRIMORDIUM



(A) Yellow host, stage 3.
(B) Distal region of red donor.
(C) Combination of graft and host.
(D) Resulting hydranth with distal region from graft and the remainder from the host.
(E) Host primordium, divided into:
(F) which became a bipolar distal and
(G) which became a unipolar distal region.
(H) A unipolar distal region arising from a distal isolate of the stage used as donor.

regenerating primordium is transplanted to the distal end of a younger whole primordium.

The material used is the form of *Tubularia* which becomes luxuriant in the harbor at Woods Hole during the warmer part of the summer. Hydranth and stem colors are red, yellow, and orange. Combinations of primordia of different colors retain a distinct color line during the differentiation phase, making it easy to distinguish graft and host. Donor and host of slightly different diameters are chosen. The perisarc of the larger can be slipped over the perisarc of the smaller until the coenosarcs meet. Fusion of coenosarcs is rapid,

TABLE 2

Stage of donor	Stage of host	Number of cases
Combinations yielding normal hydranths		
2	2	2
3	2	1
*4	2	4
*5	2	6
*5	3	4
6	2	1
Combinations in which only partial distal suppression and limited proximal development occurred		
6	2	1
6	3	2
7	3	1
Combinations in which graft and host did not cooperate to form hydranth		
2	2	6
3	2	1
5	1	20
5	4	3
5	5	3
6	1	23
6	2	3
6	3	2
6	6	2
7	1	37
7	2	5
Adult	1	44

* These three combinations always yielded normal hydranths.

and good takes are achieved in most cases. The operations and results are diagrammed in CHART 2.

If the graft is a distal piece from a stage 2 to 6 primordium on a younger differentiating primordium, stage 2 or 3, positive results may be obtained (TABLE 2). The usual course of events is the self-differentiation of the distal graft, suppression of distal differentiation in the distal part of the host, and its conversion to the proximal part of a hydranth. The presumptive proximal portion of the host is relegated to an even more proximal type of differentiation and becomes stalk of the hydranth.

Only hosts which have passed through the preliminary preprimordial stages and are ready to differentiate can be affected specifically by grafts. Host pieces from freshly cut stems do not respond. In such cases, the graft self-differentiates and suppresses all differentiation except stalk formation in the young host.

Hosts remain receptive for only a few hours. After a host has reached stage 4, just prior to the time for visible tentacle striation, it is no longer influenced by a graft at its distal end. It is now so set that it will continue to develop independently.

Likewise, only differentiating regions are capable of influencing hosts in the receptive stage. The donors must have achieved stage 2 and be no farther along than in the earliest phase of visible distal tentacle formation, stage 6.

As can be seen from TABLE 2, there were no failures in the production of normal hydranths when the grafts were from donors in stages 4 or 5 and the hosts were in stage 2 or 3.

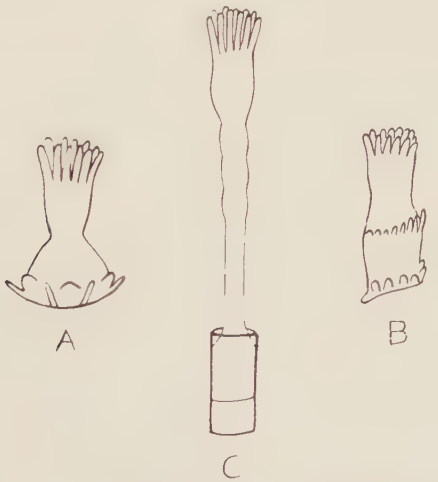
Sometimes younger grafts, stages 2 and 3, are effective in suppressing distal differentiation and thereby forcing the distal part of the host into a more proximal type of development but, more often, grafts between young donors and hosts lead to loss of organization already attained in both graft and host. This statement was true in the seven failures with young donors, stages 2 and 3, tabulated in the third part of TABLE 2.

When failures occur after older donors, stages 6 to adult, are used, one observes no loss of organization in the graft. If its host is young, stages 1 to 3, there is complete suppression of all regeneration in the host. If the host is older, stage 4 or older, it too will self-differentiate. In such case, the graft produces the distal part of a hydranth, and the host produces a whole hydranth. The result is two distal regions in tandem followed by one proximal region.

The borderline cases in the second part of TABLE 2 and in CHART 3 arise when the host is slightly too old. In *A* of CHART 3, there was suppression of the distal part of the host, but there was also a partial suppression of the proximal

CHART 3

RANGE OF RESULTS WHEN GRAFTS TOO OLD TO EXERT A CLEARLY SPECIFIC EFFECT



	Stage of distal graft	Stage of whole host primordium
A	7	3
B	6	3
C	Adult	3

(A) Complete suppression of host distal region but also partial suppression of proximal region.
(B) Incomplete suppression of both distal and proximal regions.
(C) Induced stem outgrowth and complete inhibition of all host regeneration.

region. In *B*, there was incomplete suppression of both distal and proximal regions of the receptive host. Apparently, in these borderline cases, not only is there a failing of specific inhibition, but there is an increase in general inhibition. Stage 6, in which definite proximal ridges and distal striations are apparent, seems to be the critical stage for loss of the ability to inhibit specifically. We know, from the work of Steinberg (1954), that *Tubularia* produces a general inhibitor or inhibitors which prevent migration of cells and the formation of a primordium. This inhibition might be effective in failures involving older grafts and younger hosts. However, older grafts from stage 7 and the adult stage cause extensive outgrowth of stalk from their hosts. Within two or three hours of the time an older graft, either a distal or a proximal region, is grafted to a freshly cut stem or to a primordium, there is rapid outgrowth of stalk from the host at its junction with the graft. This positive induction of stalk outgrowth by hydranth regions may preclude any specific inhibition which might otherwise be exhibited. We are not yet sure that specific inhibition stops when the period of differentiation ends. There remains some means, even in the adult hydranth, of suppressing the development of distal structures in proximal regions. As soon as distal dominance is stopped by removal of the hypostome, a new hypostome forms. There may be a one-way transmission of specific inhibition which occurs throughout life.

The work reported here involves only transplantation of distal regions to distal ends of host primordia. Additional work which will be reported elsewhere indicates that the specific inhibition works primarily in one direction. For example, distal regions grafted with normal polarity to proximal ends of whole primordia have no effect. It has also been possible to demonstrate one-way inhibition of proximal by proximal.

Rana pipiens

An attempt has been made to learn whether adult frog tissues produce substances which may specifically inhibit the differentiation of like embryonic tissues and organs.

The procedure was, first, to remove most of the jelly from developing frog eggs with scissors and watchmakers' forceps. The eggs were then cultured with bits of brain or heart or blood. They were treated, during cleavage, blastulation, and early gastrulation, in full strength Holtfreter's solution containing bits of brain or heart or whole blood. The culture fluid for the tissues and eggs contained one to five milligrams of chloromycetin per liter. This fluid was used only during the period when the eggs and tissues were cultured together. These amounts, used under the conditions of this experiment, have no effect on the development of the eggs. Treatments were started at the 32- or 64-cell stage and continued for three to five days at 9° to 12° C. until the dorsal lip had appeared as a slight groove or, sometimes, until the lip had become a crescent. The eggs and tissues were cultured in 4.5 cm. Stender dishes containing 10 cc. of the culture fluid. Each dish held 7 to 15 eggs. For treatment with brain, one adult brain cut into approximately 30 pieces, was used per dish. One heart, also cut into approximately 30 pieces, was used

per dish. The blood dishes received 8 to 10 drops of fresh whole blood which was allowed to clot in a few cc. of the culture solution. After the clot had formed, more solution was added to bring the total to 10 cc. Eggs were added so that they rested on the clot at the bottom of the dish. Every day, during the three to five days of treatment, the eggs were transferred to fresh culture medium containing fresh tissues.

In this way, it was possible to have the eggs developing slowly for several days in a medium containing products of living tissues. That the tissues do remain alive and functioning can be seen, in the case of the heart, from the fact that atrial pieces continue to beat for the 24 hours they are used. There was very little hemolysis of the blood, but clots often softened during the treatments. Brain pieces remained firm with no apparent signs of degeneration. If tissues were allowed to die during a run, the eggs also died or developed very abnormally.

In the experiments reported upon here, all of the treatments were terminated during the early gastrula stage. The gastrulae were then removed from the solutions containing the tissues and cultured, thereafter, in 20 per cent Holtfreter's solution or in dechlorinated tap water at 18° to 20° C. or, in some cases as noted below, at 20° to 23° C. The volume of solution after treatment was increased so that each egg from the gastrula stage onward had approximately five cc. of solution.

If treatments are not begun until a late gastrula or early neurula stage, the eggs remain unaffected and will develop on time and without defects. For example, the neural system will form in embryos, even when they are surrounded with a medium containing bits of brain if the tissue is not added until a small yolk plug stage.

The pregastrular treatments lead to quite variable effects. In 12 experiments, the treated eggs showed a drop in rate of development which was already apparent at the end of treatment in the early gastrula stage, or the drop became apparent somewhat later during gastrulation. These eggs either stopped developing as gastrulae or, if they continued further, showed gross general abnormalities which made a study of specific defects impossible. In 10 other experiments there was no appreciable effect of the treatments. The eggs were not delayed, and over 90 per cent developed normally. Most defective embryos showed general defects which were found as frequently in controls which were treated identically, except that they were not cultured with tissues. Only a few cases of what might have been specific effects were noted in these 10 experiments.

There were only four experiments in which clearly specific defects were observed. In these experiments, the eggs did not suffer a lag in development during the treatments or during gastrulation. There was delay later, which varied in all four experiments according to the tissue used. The data on delay and specific inhibition in these four experiments are tabulated (TABLES 3 and 4).

Eggs treated from cleavage stages to early gastrulation, if treated with brain, developed normally until the time of neural plate formation, and then did not change appreciably for several hours to three days. Those which remained as

TABLE 3

Treated with	Reaching larval stage	Stage at which development retarded			No retardation
		Neural plate	Neural tube and tail-bud	After tail-bud	
Brain	43	36	0	0	7
Heart	58	8	37	0	13
Blood	174	4	8	126	36

TABLE 4

Treated with	Reaching larval stage	Defects limited to				General defects	No defects
		Neural system	Vascular system	Heart and vascular system	Heart		
Brain	43	5	0	0	0	10	28
Heart	58	0	0	0	8	13	37
Blood	178	0	59	23	0	14	82

early neurulae as long as three days recovered to the extent of completing neurulation but showed gross defects and died in a few days. Most of the brain-treated embryos delayed as neurulae for one or two days either exhibited specific neural defects or recovered completely and developed with no defects. The individual differences in amount of delay in the brain-treated series lead to a great spread of developmental stages after recovery. Four brain-treated embryos, all from the same dish, are shown in FIGURE 2. The one on the left showed no delay and developed normally. The others were delayed for varying periods during neurulation but had recovered and had resumed development. In FIGURE 4 are pictured six other brain-treated embryos from another experiment. All but one, the middle one on the left, show irregularities in the cephalic region. It is tempting to suspect that the vesicles and the roughened epidermis over the brain region are the result of specific effects on the brain. This region of the body, however, is peculiarly sensitive to many generally toxic substances. It may be that the greater number of cases showing this type of defect after treatment with brain resulted from generally toxic agents which were in greater concentration in the brain cultures. Such cases are not used in the specific defect column of TABLE 4.

Only those embryos which showed more local defects were used. Sections of three such embryos are shown (FIGURES 5 to 7). In these cases, the only defect observed is poor morphology of the central nervous system. The arrangement of cells is more random than expected, and a neural canal is incomplete or missing. All of the other tissues and structures of the embryos appear to be normal. These defects, which are confined to brain-treated embryos, are the only ones considered to be specific. The time of delay, in the early neural plate stage, also appears to be specific.

FIGURE 1. Two heart-treated tadpoles. The one on the left is unaffected, while the one on the right is edematous in the heart region and shows the typical heartless syndrome.

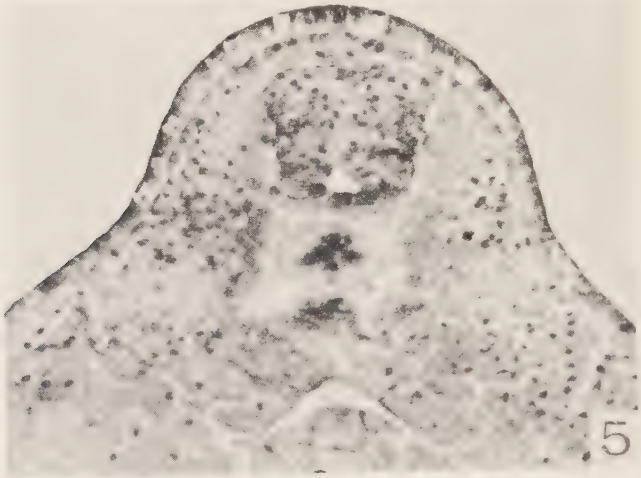
FIGURE 2. Four brain-treated embryos. The one on the left was not delayed. The other three were delayed in the early neural plate stage but recovered at different times.

FIGURE 3. Two blood-treated tadpoles. The one on the right is unaffected. The one on the left is bloodless and is lagging behind its partner.

FIGURE 4. Six brain-treated embryos. The one on the left in the middle is normal. The other five show either roughened epidermis or vesicles in the head region.

A neural abnormality which is common during recovery of the brain-treated embryos results from uneven recovery. Often, one neural fold will be much larger than the other. A section of such an embryo after fold closure is shown in FIGURE 8. Recovery from this condition may be complete with no asymmetry noticeable after one week.

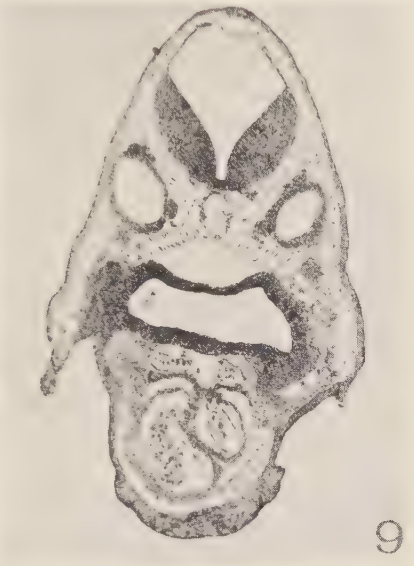
In FIGURE 1 may be seen two heart-treated embryos. The one on the left is normal, while the one on the right is edematous in the heart region and the edema has spread laterally and posteriorly. In all of the heart-treated em-



FIGURES 5 to 7. Cross-sections through brain-treated embryos showing a solid or almost solid central nervous system in which the cells are abnormally random in position. All other structures are normal.

bryos showing heart abnormality, there is localized edema becoming more general and subsequent retardation of growth. Especially noticeable is the failure of outgrowth of the external gills and the tail. These defects had been noted earlier when heart rudiments were removed surgically (Knower, 1907; Kemp, 1953).

In FIGURE 9 may be seen a cross-section through the gill and heart region of a normal young larva. Similar sections of heart-inhibited larvae are shown



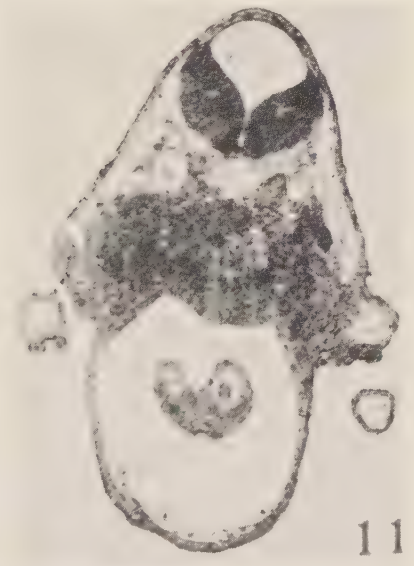


FIGURE 8. A cross-section through a brain-treated embryo shortly after the neural folds closed. This embryo had been retarded as a young neurula and had recovered asymmetrically.

FIGURE 9. A cross-section through the heart region of a normal embryo.

FIGURES 10 and 11. Cross-sections through the abnormal hearts of two heart-treated embryos.

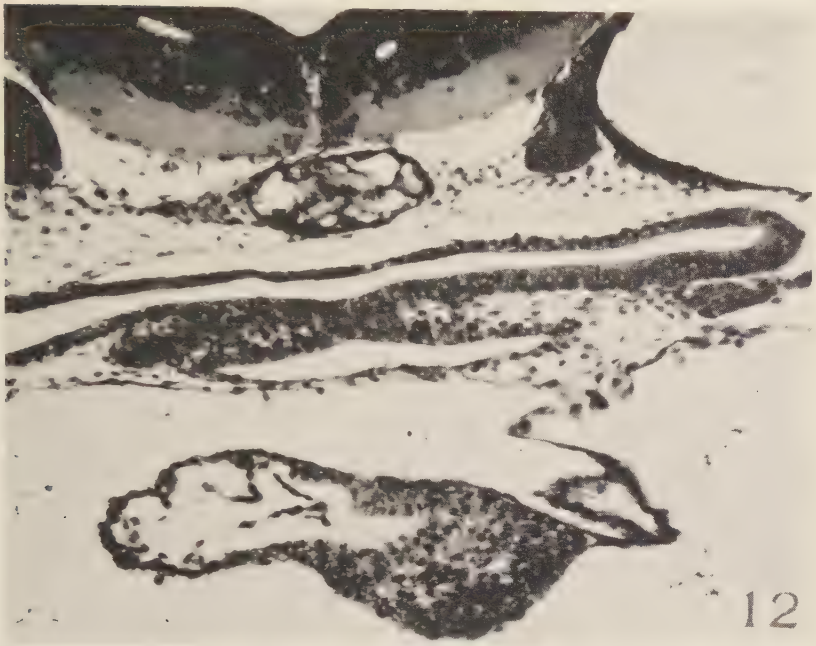
in FIGURES 10 and 11. The pericardium has enlarged, but the heart has remained small and too solid, with the cells not spreading out to form chambers and the divisions between them. The normal *S* shape is not attained by such hearts. In two cases, however, a heart which had been retarded so that it was several days behind the rest of the larva did recover and formed a good functional heart from the delayed rudiment. This phenomenon happened only when the affected larvae were kept at 12° C. At 18 to 20° C., these tadpoles soon show general defects in the absence of a functional heart.

In FIGURE 13 may be seen a much inhibited heart. In the center is the undifferentiated heart, with cells too large and too yolky. That this condition is a specific defect is indicated by the well-developed appearance of the adjacent tissues, pharyngeal epithelium above, and striated muscle to the right and below.

The blood-treated embryos develop quite normally and at a good rate, often somewhat more rapidly than controls, until Shumway's stage 18 or 19. At this time, some lag is noted in many cases and, when external gills are growing out and blood cells beginning to pass through them (stage 20), a quantitative defect is observed. The gills are very small, and the number of blood cells passing through them in a given time varies from the normal number to zero. Later, when the ventral body wall is clearing, most of the completely bloodless tadpoles show good beating hearts with no cells passing through. The general morphology of bloodless tadpoles, kept at 12° C. after the initial defect was noted, is very good. In FIGURE 3 may be seen two tadpoles which had been cultured with blood until gastrulation. The one on the right had not been affected. The one on the left is bloodless and, as can be seen, is somewhat retarded. The affected one had no external gills, its intestine had not yet coiled, the tail fins had not expanded as much and had not cleared, and the closure of the operculum was delayed. This syndrome is usual in bloodless tadpoles.

A section through a tadpole which had no blood passing through its well-formed, beating heart is shown in FIGURE 16. This tadpole, kept at 12° C. from the time a blood defect was first noted, continued to develop slowly and, like many others, did form blood eventually. This tadpole, at the time of fixation, still had no blood cells in circulation, but it was recovering from the inhibition, and blood islands, red in color, could be seen in various parts of the body. The most common places for late blood islands to appear in these tadpoles are ventral pericardium, ventral and lateral peritoneum, walls of lymph spaces, atrial walls, gills, and the tail. The tadpole of FIGURE 16 has a blood island in the parietal pericardium (indicated by an arrow). In a higher power view, another blood island of the same larva can be seen in the ventral peritoneum. Apparently, blood cells have differentiated, but endothelial connections between islands are incomplete. Only later are the vessels completed in the formerly bloodless tadpoles. FIGURE 15 is a section of a formerly bloodless tadpole which had recovered at 12° C. At the time of fixation, six weeks after treatment, it had many blood cells, including some visible in its heart.

Recovery from a defect which begins as a specific failure of blood islands to differentiate is possible only at lower temperatures. At around 20° C., there



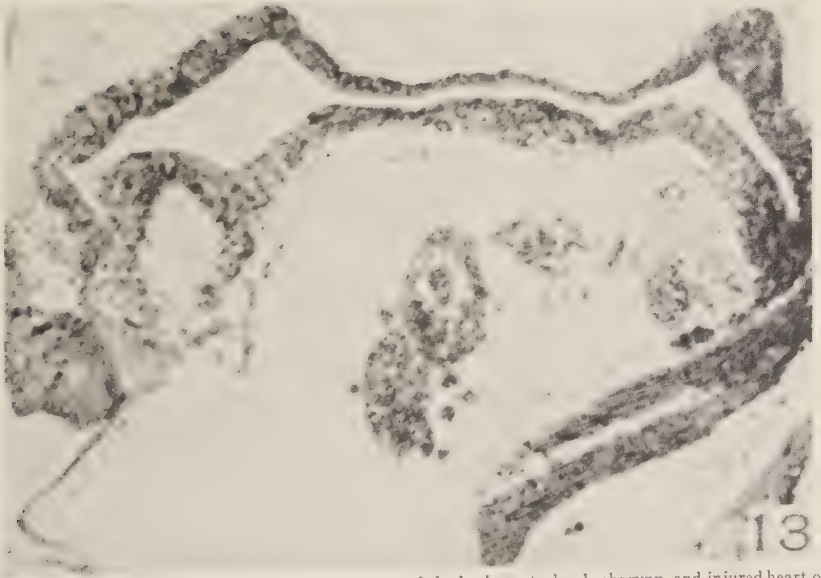


FIGURE 12. A cross-section through the lower part of the brain, notochord, pharynx, and injured heart of a blood-treated tadpole.

FIGURE 13. An undifferentiated heart rudiment surrounded by well-differentiated pharyngeal epithelium and striated muscle in a heart-treated tadpole.

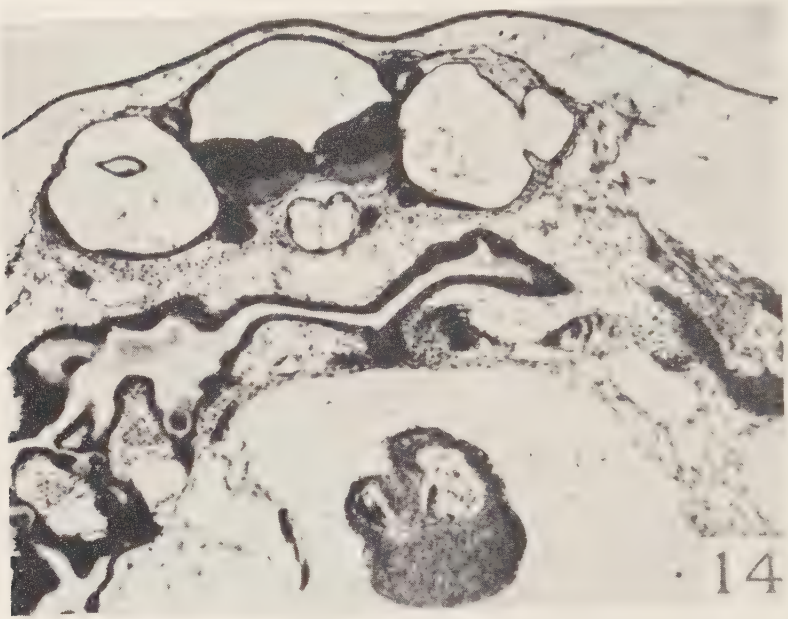
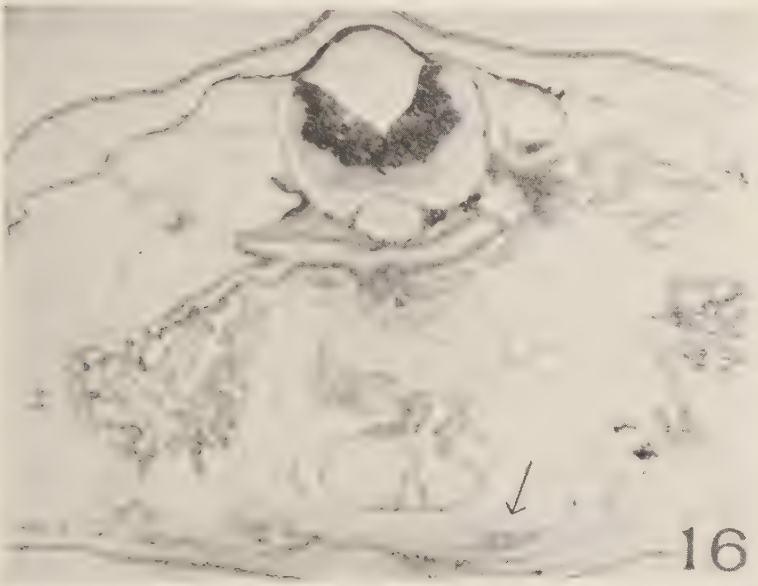


FIGURE 14. A cross-section through the injured heart of a bloodless tadpole kept at 20° C. after its pregastrular treatments with blood.

FIGURE 15. A cross-section through the normal heart of a bloodless tadpole kept at 12° C. for six weeks after its pregastrular treatments with blood. This tadpole had recovered completely and had produced blood cells. Some are visible in the heart.



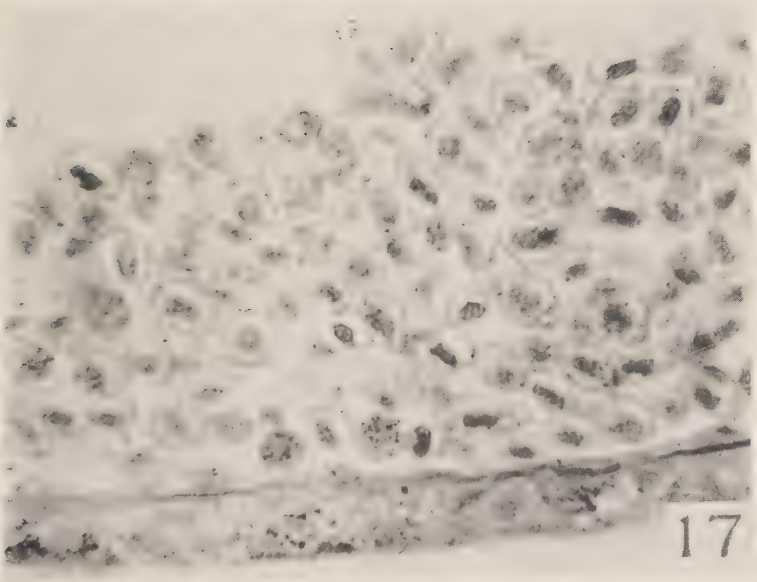


FIGURE 16. A cross-section through a blood-treated tadpole which had developed quite normally except for its lack of blood. At the time of fixation some blood had formed and its position in the pericardium is indicated by the arrow.

FIGURE 17. Blood cells in the peritoneum of the same tadpole of FIGURE 16.

are widespread failures in all internal systems. The gut changes very slowly, and hearts begin to fail. FIGURE 14 is a section through the heart region of a blood-treated animal kept at 20° C. after its treatments. It was observed daily and had been recorded as having a well-proportioned heart with a normal beat. This bloodless tadpole developed quite well, as can be seen in the photograph, but its heart, which had been normal, became smaller and too opaque and, in a few days, began to fibrillate. In another day there was no more contraction observed. At this time the animal was fixed. The heart of a similar case is shown in FIGURE 12. Here, too, a functional heart in a bloodless embryo had failed and is seen, in section, to be too small and to have the typical collapsed ventricular structure.

These affected hearts in blood-treated animals are very different from those affected by culture with heart. They do differentiate well and become functional on time. Subsequent failure of the heart along with general growth retardation probably result from a lack of blood. Hearts have been observed to fail in bloodless tadpoles at 20 to 23° C., but not at 12° C.

In the 10 experiments in which no delay in development at any stage was observed, there were no, or at most very slight, specific defects. In another 12 experiments, the effects on the embryos were too great and widespread. In these experiments, retardation was observed by the end of the treatments or somewhat later during gastrulation. It was only in those four experiments, in which an apparently specific delay period was noted, that the clearly specific morphological effects were observed.

It appears that one or more factors not under the control of the investigator determine whether or not the solutions will be effective. There is also always the possibility that the treatments will kill the eggs before the time for specific effects. In the few experiments which have been useful, there have always been some animals showing gross abnormalities, some not affected and some showing specific defects (TABLE 4). Never have treated eggs of any group shown the specific effects found in tadpoles from either of the other two treated groups. Although variables not under control often prevent specific effects, when they do appear they are extremely specific. It is only a narrow intermediate zone between too little and too much which is useful to us.

It is not known what the factor is that enables the solutions to affect the embryos in some experiments and not in others. It is suspected that, in those cases in which no effect is obtained, the test solutions have not penetrated the cells. In three of the four useful experiments, it appeared that the eggs were sensitive to our Holtfreter's solution. Controls kept in this solution at low temperature until gastrulation were adversely affected, and many developed abnormally. At the same time, eggs from the same batch developed normally in dechlorinated tap water. In these experiments, the tissues seemed to have a protective action against the salt solution, for there were many more general abnormalities among the control embryos than among the tissue-treated ones. It may be that an injury factor is involved, and that it is only when the surface of the egg is injured that the cells can be affected with our solutions. This inference tallies with the demonstration that surface-coated ectodermal cells are unaffected by various things which will induce them to differentiate when they

are not coated. It also happens that three of the four experiments yielding specific inhibition were started in December or January. The fourth was an October experiment, in which visibly poor eggs were used. The October eggs were small and dark, and they showed very little of the white vegetal area. This type of egg is obtained from underfed females. It appears, then, that batches of eggs differ in susceptibility, and that their susceptibility may vary with the time of year. It is also possible that the tissues vary in their potency with the time of year.

Conclusion

The work reported here seems to support the hypothesis that pattern formation may result, in part, from specific inhibitory effects proceeding from more developed to less developed regions. Very similar ideas have been expressed by other investigators as the result of work with quite different organisms (Child and Watanabe, 1935; Braun, 1952; Lüscher, 1953).

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Discussion of the Paper

Doctor Alexander G. Karczmar (*Sterling-Winthrop Research Institute, Rensselaer, N. Y.*): I think that the idea of inhibitory regulations emanating from the more mature to the less mature embryonic regions is so tempting as very possibly to be true. It is also encouraging to see that the lesions described by Doctor Rose are site-specific. This fact is important because I think that most of us have noticed nonspecific inhibitory and even toxic, probably diffusible, factors present in the tissues. In some cases, their effect can be measured, as in the case of naturally occurring tissue anticholinesterases recently postulated by us (Karczmar and Koppanyi, Fed. Proc., **13:** 372–374, 1954). In our case, these factors, residing in one adult tissue, appeared to be inhibitory to the enzymic activity of any other tissue. Thus, there was little specificity in their action.

There is another general possibility for explaining the mechanism of inhibi-

tion of immature by mature tissues, besides postulating inhibitory substances, and that is the possibility of absorption by the tissues already differentiated of the inductors necessary for the subsequent differentiation. For instance, Zalokar* caused inhibition of Wolffian regeneration by means of implanting the crystalline lens, and presented evidence that the lens adsorbs the refinal inductor. Obviously, the lens may lend itself better to such a role than the hydranth regions or the living adult tissues used by Doctor Rose. I wonder, however, whether this mechanism of inhibition by adsorption of prerequisite substances cannot be considered even in these cases.

Doctor Rose: I do not wish to leave the impression that inhibition occurs only in one region after the treatments There was general inhibition of development in every case where specific inhibition was observed. Representative cases are shown in PLATE 1. The general inhibition is followed by recognizable specific inhibition only when the inhibition is more lasting, but still temporary, in the particular embryonic organ. In view of the demonstration by Doctor Ebert, in another paper in this series, that cardiac myosin is detectable in early stages throughout the blastoderm and is only later limited to the heart, I wonder whether we may be dealing with a general phenomenon Possibly reactions leading to specific differentiation are more general than we have thought, the differentiation involving progressive loss of abilities until finally the reaction occurs in only one region.

The question about inhibitors versus inductors is an intriguing one. From the work of Zalokar on the lens, one can understand, as Doctor Karczmar suggests, that an already formed structure might be adsorbing the inductors. This view seems to be ruled out by the later work of Stone and Vultee† who have demonstrated that fluid from an eye with a lens will prevent regeneration of lens from iris. If an inductor adsorbed by lens is involved, the fluid from the eye with a lens should contain very little inductor. But, in keeping with the assumption of an inductor, the recipient eye minus a lens should be unaffected by the absence of inductor, which it can itself produce. A lensless eye without the injection does produce a lens and presumably has the necessary hypothetical inductors. This appears to be a phlogiston situation. The only interpretation which seems to fit the more recently acquired facts is that not the absence of an "inductor" but the presence of specific lens inhibitor prevents lens formation from iris.

Doctor R. L. Bacon (*Department of Anatomy, John Hopkins University, Baltimore, Md.*): I should like to comment on Doctor Rose's experiments with heart. During my graduate work, in the course of attempting to obtain successful development of hearts from explanted presumptive heart mesoderm of *Amblystoma*, it occurred to me that possibly more successful results might be had if I used Holtfreter's solution, in which mesodermal tissues had been growing for a time, to "modify" or "condition" the medium. I found that a medium in which older larval hearts had been growing was the only one in which young heart explants did *not* grow successfully. This test was not of itself a crucial experiment but, at least, it indicated to me that this method was

Zalokar. Revue Suisse de Zool. 1944. 51: 443–521.
† Stone, L. S. & J. H. Vultee. 1949. Anat. Record. 103: 144–145; Stone, L. S. 1953. Am. J. Ophthalmol. 36: 31–39.

an unsuitable technique for culturing embryonic hearts and, therefore, the procedure was discontinued.

Doctor J. Philip Trinkaus (*Yale University, New Haven, Conn.*): Did you use other mesodermal organs?

Doctor Bacon: Yes. Hearts differentiated satisfactorily in media which had previously contained pronephros or somite.

Doctor A. M. Schechtman (*University of California at Los Angeles, Los Angeles, Calif.*): As I see it, the critical point involved is whether certain specific defects may be attributed to treatment of the early embryos with certain specific kinds of tissues or organs. After treatment with a given tissue, for example blood, we find that later stages of the organism show (1) no effects or (2) homologous defects or (3) heterologous defects. Considering the numbers of embryos treated and the relative small number which show homologous defects, I wonder if one can feel fairly certain that such effects occur in great enough number, and are sufficiently associated with homologous as compared with heterologous treatments, to establish specific inhibition by the homologous treatment? In other words, since defects of the heart and blood occur after a great variety of conditions, is the percentage of defects produced by treatment with heart and blood sufficiently great to warrant the conclusion that homologous defects are at the same time specific defects? Is there a possibility that the concentration of tissue or organ proteins, *etc.* around the embryos during treatment varies sufficiently so that the variations in numbers or types of defects can be due to some general factor such as osmotic pressure or degree of anaerobiosis rather than to heart or blood as such?

Doctor Edgar Zwilling (*Storrs Agricultural Experiment Station, The University of Connecticut, Storrs, Conn.*): Although I am not a statistician, it seems evident that such variable material cannot be handled by simple statistics. One must utilize suitable variance analyses for such data. I feel certain that a situation in which only five or so embryos in an experimental group develop a unique syndrome is significant when this syndrome never appears in controls. This should be true even though 'reactors' do not occur in every experimental group.

If one recalls the data from experimental teratogenesis, one is not surprised at the variability of results from Rose's experiments. There are ample, well-substantiated data from avian and mammalian material which demonstrate that responses to various treatments which elicit teratogenic reactions differ considerably with the breed or strain of animals used for the experiment. These variations in susceptibility have a genetic basis. In addition, there are well substantiated cases in which seasonal variations in response of offsprings from a single group of animals have been demonstrated. There are also variations which are conditioned by the nutritional state of the parents of the embryos under investigation. It is not very surprising, in view of this information, that Rose, working with frogs whose genetic and nutritional status is unknown, should encounter considerable variability in his results. In such work it is important that each experiment should contain its own control, and that the controls be consistently free of the specific anomalies encountered in the experimental groups.

Doctor Rose: It certainly is true that defective embryos can be obtained after a number of treatments including disturbances in ionic concentration and after partial anaerobiosis. Such defects, as far as I know, are never limited to one organ. From my own experience and from the literature on the subject, I cannot recall anything like the circumscribed defects pictured here. The numbers are small but certainly significant for the reason which Doctor Zwilling points out. There were 5 embryos showing only brain defects after culture with brain, 8 showing only heart defects after culture with heart, and 59 after culture in blood with only blood defects of which 12 were completely bloodless. There were no defects in single heterologous organs. This is a situation in which a very simple statistical procedure is applicable. Each time a single defect occurred there was, on a chance basis, the possibility that it would occur in either one of the two heterologous organs or in the one homologous organ. After elimination of all doubtful cases, there were 25 cases in which the defect was in the homologous organ. There were no limited heterologous defects, not even doubtful ones. One would expect this result in only $^1/_{3^{25}}$ of the cases.

It was suggested during the discussion, by someone whose name cannot be recalled, that the negative experiments should be included in the analysis of probability. This is irrelevant if one is trying to determine whether the effect is specific when it occurs. Obviously it does not occur in all experiments. There is no reasonable way of changing the probability of a specific event by adding to an analysis of probability experiments in which no specific defects occurred. It seems quite clear that some factors not under the control of the investigator are operative. Possibly, as suggested by Doctor Zwilling, genetic factors play a large part. This is to be expected if the hypothesis behind this work is correct. It is equally clear that when organ-limited defects occurred they were in the homologous organ. That is the point of the work on the frog embryo reported in this paper.

Doctor James D. Ebert (*Department of Zoology, Indiana University, Bloomingdale, Ind.*): In recent years, few preliminary statements of findings have aroused the interest of students of development as greatly as did Doctor Rose's account of his results before the American Society of Zoologists in 1952. The conference on which this monograph is based has provided the first opportunity for many of us to hear the details of these studies. The history of attempts to formulate a general theory of differentiation and growth testifies both to the need for a conceptual framework for the increasing store of diverse data, and the difficulties that have barred the way. Hence, it is encouraging to learn that Doctor Rose has continued to seek a critical test of the hypothesis advanced in 1952, that differentiation proceeds as more rapidly developing regions suppress like development in more slowly developing adjacent regions and, thereby, channel the slower regions into another kind of differentiation. I have been keenly interested in the course of the investigation, since it bears directly on two lines of research in progress in my own laboratory. For example, the pattern of differentiation of cardiac myosin, discussed earlier at this conference, suggests that the localization of this specific protein may be due to a failure of synthesis in areas adjacent to the heart-forming regions in which a high level of synthesis is maintained. It should be possible to test this hypothesis by determining

quantitatively the rate of synthesis of cardiac myosin in defined regions of the early embryo, and to analyze the effect on the synthetic process of experimental interventions of the type described by Rose. In addition, for the past six years, our group has been engaged in an experimental analysis of the concept of organ-specific control of growth. This concept, proposed initially by Doctor Paul Weiss, centers on the question as to whether organ-specific compounds, instrumental in the synthesis of a given protoplasm, occur in complementary pairs. It amplifies and extends the earlier theoretical statement of Jacques Loeb, whose ideas are embodied also in Doctor Rose's arguments. Since I have discussed the history of the concept and the progress in our own laboratory only recently (Thirteenth Symposium, Society for the Study of Growth and Development), I shall not review our findings at this time.

Much of the discussion of Doctor Rose's paper centers on the lack of consistency in the findings. It is on this aspect of the work that I wish to comment. First, I wish to reiterate the opinion of Professor Schechtman, who called for a complete statistical analysis of the data. Let me emphasize that I do so because I believe that such an analysis might enable us to make a more effective evaluation of the possible sources of experimental inconsistency which have made a critical test of the hypothesis so difficult. I have no doubt of the possible significance of the positive findings. It is impossible to gainsay the results presented in the histological sections. From the discussion, however, I am not clear as to the extent to which the results vary (1) throughout the entire course of the work, *i.e.*, from experiment to experiment, and (2) within a given experiment. Are the positive findings scattered throughout the course of the investigation, or are they limited to a relatively few experiments? Doctor Rose briefly touched on the question of seasonal variation in the findings. I am not clear, however, whether positive findings have been obtained in experiments carried out during the normal breeding season of the frog. Have experiments been performed employing eggs from frogs captured during the breeding season or fertilized eggs taken fresh from natural ponds? If not, to what extent can the results be attributed to the use of eggs from frogs which have been kept in the laboratory for some time? Is it possible that eggs taken from starved females are hypersensitive to the responsible factors (cf. Spence, Bieber, Hitchings, and Charipper, Anat. Rec., 120: 713)? Have aged or overripe eggs been employed in any experiments? If the possibility that the source of variability is the egg itself can be ruled out, at least in so far as seasonal differences in egg content and state of maturation are concerned, one is led to search for sources of variability in the experimental procedures. In my opinion, the most likely source of variability may lie in the condition of the "bits of living adult tissues" in which the pre- and early gastrula stages are cultured. Doctor Rose has suggested that he does not believe that autolysis of the tissue fragments is important. My limited experience would suggest that the contrary might be true. Therefore, I am led to inquire whether cytological analyses have been performed on those tissues which have been shown to exert a specific inhibitory effect. Are these tissues carrying on a normal metabolism? It is interesting to note that several of the investigators who have failed to find critical evidence of a similar nature using other experimental materials

have attempted to maintain the tissue implants in a normal physiological state. The integrity of the tissue has been emphasized. For example, in our laboratory we have studied the effects of grafts and homogenates of late embryonic and adult chicken spleen on the early development of the host spleen. Following intracoelomic transplants of spleen and the injection of spleen homogenates into 66- to 72-hour embryos, the host spleen has made its appearance at the usual time (the end of the fourth day of incubation) and has differentiated normally. It is possible that the critical point may be that the effective cases are these in which the responsible factors are produced or released as a result of the breakdown of a more stable component. It may be helpful to think of the effective substance as a *modified* tissue-specific metabolite. Have experiments been performed in which tissue fragments have been allowed to autolyze for varying periods of time, and the autolyzed products tested for their effectiveness? Have media conditioned by prolonged periods of cultivation of adult fragments been employed?

Doctor Rose: As stated in the answer to Doctor Schechtman, I do not agree that the findings are inconsistent. The findings are very consistent, and I think there is overwhelming improbability of their arising by chance. We may be confusing two quite different kinds of consistency. The data indicate a striking consistency in that local retardations in differentiation have only occurred in the same organ or system from which the adult tissue was taken. It was also found quite consistently in all experiments where specific effects were found that some embryos did not respond and that there were other batches of eggs which were completely unaffected by the treatments. The claim is not made that it is known how to affect all or most eggs. What is claimed is that when the rare local effect is obtained it is specific. I think that some of us may be summing up two kinds of consistency, the specific effect and the rarity of its appearance, and concluding therefrom that the result is inconsistent.

I wish that we knew why the very consistent specific effect comes in some experiments and why there is no effect in others. I have run a number of experiments in which a variety of factors which might "open" the egg, or make it more susceptible, or enhance the effect of the tissue have been tried but nothing has been learned. It would be well to have a more reliable assay method. This situation is reminiscent of the period of adrenal study before cortisone was known. Although the need for and some of the effects of cortical tissue were known, it was not until the hormone and its effective quantities could be determined that the picture cleared.

I had hoped that before presentation of this material, the consistency of which was not in doubt, there could be devised a better assay for the testing of various substances from different tissues. As Doctor Briggs has suggested, and in the light of Doctor Bacon's discussion, I now think that work with explants is more promising. Doctor Niu's elegant method, which obviates the surface coat barrier, should prove most useful in future studies.

Doctor Ebert asks many questions. I can say very little more of value than is reported in the paper. In three of the positive experiments, perfectly good eggs were used. These were large with wide white fields, and from them

almost 100 per cent of the untreated eggs developed normally. Eggs used in one of the positive experiments were from a female that had apparently been starved. The eggs were not over ripe in the sense that they had remained in the uterus too long. Instead, they were the type one finds after starving the female. Such eggs are smaller, have a very limited white field, few are fertilizable, and the eggs are more sensitive to poor conditions during development. I have never used fresh eggs from natural ponds. Most of the frogs were sent parcel post from Alburg, Vt., and kept at 4° C., their natural hibernating temperature, until ovulation was induced by injection of several pituitary glands

I do not know, as Doctor Reiner suggested, whether there is much value at this time in discussing whether the substances involved came from cells before or after death. I tried to work with living pieces of tissue. In some experiments where the tissue did die, the eggs died also. Possibly a very limited cytolysis is necessary. Such a cytological study would have to be performed by someone with different bents than mine.

Reference was made by Doctor Ebert to similar experiments by others which have failed. Without knowing the details of the experiments, it is impossible to assess them. I do know of several important considerations. In my work, it has been important to work before gastrulation and, in order to avoid widespread tissue destruction in the embryos, it has been necessary to work at low temperatures. With our present knowledge, attempts to repeat this work at high temperature or after gastrulation has started would seem predestined for failure.

Fortunately, I think, we can avoid the question of priority for any living person for the concept of organ-specific control of growth. The concept was developed in the writings of several people before the present era of induction studies and gave way, at least temporarily, in the intellectual ferment of the time in which we grew up.

I wish I could answer most of Doctor Ebert's questions, but some of the questions must await further experimentation.

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